Duchenne and Becker muscular dystrophies: a review of animal models, clinical endpoints, and biomarker quantification

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Abstract

Duchenne and Becker muscular dystrophies (DMD/BMD) are neuromuscular disorders that primarily affect boys due to an X-linked mutation in the *DMD* gene, resulting in reduced to near absence of dystrophin or expression of truncated forms of dystrophin. Some newer therapeutic interventions aim to increase sarcolemmal dystrophin expression, and accurate dystrophin quantification is critical for demonstrating pharmacodynamic relationships in preclinical studies and clinical trials. Current challenges with measuring dystrophin include the variation in protein expression within individual muscle fibers and across whole muscle samples, the presence of pre-existing dystrophin-positive revertant fibers, and trace amounts of residual dystrophin. Immunofluorescence quantification of dystrophin can overcome many of these challenges, but manual quantification of protein expression may be complicated by variations in the collection of images, reproducible scoring of fluorescent intensity, and bias introduced by manual scoring of typically only a few high-power fields. This review highlights the pathology of DMD/BMD, discusses animal models of DMD/BMD, and describes dystrophin biomarker quantitation in DMD/BMD, with several image analysis approaches, including a new automated method, that evaluates protein expression of individual muscle fibers.

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Introduction

Muscular dystrophy

Muscular dystrophies are a large, heterogeneous group of diseases that result in progressive weakness and degeneration of the skeletal muscles that control movement (Guiraud *et al.*, 2015, Kornegay, 2017, Wicklund, 2013). Muscular dystrophy was first described in the early 1800s, and since then, many different forms have been identified, caused by an expanding list of genetic mutations (Kamdar and Garry, 2016, Tyler, 2003). More than 30 types of muscular dystrophy have been identified, and examples include Duchenne/Becker (DMD/BMD), myotonic, limb-girdle, facioscapulohumeral, oculopharyngeal, and Emery-Dreifuss (Berger and Currie, 2012, Plantie *et al.*, 2015). The genetic mutations, major muscle groups affected by each mutation, clinical syndromes, and treatment options have been reviewed elsewhere (Bastian *et al.*, 2015, Bonne and Quijano-Roy, 2013, Chau and Kalsotra, 2015, Fanin and Angelini, 2015, Gatica and Rosa, 2016, Luigetti *et al.*, 2015, Madej-Pilarczyk and Kochanski, 2016, Meola and Cardani, 2015, Wang and Tawil, 2016, Wicklund, 2013). This review will focus on DMD and BMD.

Duchenne and Becker Muscular Dystrophies

DMD and BMD are X-linked disorders that have a combined estimated prevalence of 1 in 7250 males (Romitti *et al.*, 2015). DMD affects approximately 1 in 3500–5000 live male births worldwide (Emery, 1991, Guiraud *et al.*, 2015, Kamdar and Garry, 2016, Mendell *et al.*, 2012, Nigro and Piluso, 2015, Robinson-Hamm and Gersbach, 2016, Romitti *et al.*, 2015). Affected boys appear clinically normal at birth; however, signs and symptoms become apparent and a diagnosis is made by around 4–5 years of age (Guiraud *et al.*, 2015, Mendell *et al.*, 2012, Robinson-Hamm and Gersbach, 2016). Functional improvements due to natural growth are observed in boys younger than age 7, until the characteristic degeneration and loss of muscle tissue outpaces maturational development and physical growth (Mazzone *et al.*, 2013, McDonald *et al.*, 2010). As muscle deterioration overtakes muscle growth, patients with DMD have difficulty walking during childhood and become wheelchair dependent by their early teens(Bello *et al.*, 2016, Flanigan, 2014, Guiraud *et al.*, 2015, Kole and Krieg, 2015, Mazzone *et al.*, 2011, Nigro *et al.*, 1983). These patients continue to lose upper extremity function and require progressively more assisted ventilation during the teenage years (Guiraud *et al.*, 2015). Cardiomyopathy is typically present by 14–15 years old (Viollet *et al.*, 2012). Premature death typically occurs by 30 years of age, though recent improvements in management of cardiorespiratory function are improving life expectancy (Guiraud *et al.*, 2015, Kohler *et al.*, 2009, Nigro and Piluso, 2015, Zaharieva *et al.*, 2013). BMD is milder and occurs less frequently than DMD, affecting 1:11,500–19,000 males (Kamdar and Garry, 2016). Typically, BMD manifests later in life with a mean onset of 12 years, loss of ambulation is delayed until the third decade of life, onset of cardiac involvement is variable, and the overall life expectancy is longer compared with DMD patients (Bushby *et al.*, 1993, Finsterer and Stollberger, 2008, Flanigan, 2014, Nigro and Piluso, 2015). Though the disease is X-linked, 3–8% of female carriers can present with symptoms ranging from mild (symptomatic carriers) to rapidly-progressive DMD-like muscular dystrophy due to skewed inactivation of X chromosomes (Guiraud *et al.*, 2015, Miyagoe-Suzuki *et al.*, 2017).

DMD/BMD Gene Mutations

DMD/BMD results from an inherited or spontaneous mutation in the DMD gene (Kamdar and Garry, 2016). Approximately 1/3 of cases arise from spontaneous mutations (Nardes *et al.*, 2012, Selsby *et al.*, 2015). The correlation between mutation and phenotype is better explained by the reading frame theory of Monaco (Monaco *et al.*, 1988). This theory argues that if a deletion leads to a shift in the open reading frame, then premature termination results in a truncated protein (or no protein), which is often associated with the DMD phenotype (Blake *et al.*, 2002). By contrast, if a deletion does not result in a frameshift, then a partially functional, abnormally small dystrophin protein is often produced, resulting in the BMD phenotype (Blake *et al.*, 2002). While the reading frame theory of Monaco explains >90% of cases, some cases contradict this model (Koenig *et al.*, 1989). In this study, there were patients with milder phenotypes who had out-of-frame deletions of exons 3–7 and there were patients with deletions that did not disrupt the reading frame who exhibited more severe phenotypes; some of these latter patients had deletions large enough to likely result in a protein too small to be functional (Koenig *et al.*, 1989). These contradictory cases suggest that there may be additional modifiers influencing the patient's disease phenotype.

A variety of *DMD* mutations have been documented. Approximately two thirds are large deletions or duplications detectable by microarray analysis, while approximately one third are point mutations or small insertions/deletions detectable by gene sequencing; less than 5% of *DMD* mutations are in the non-coding regions of the gene (Gee *et al.*, 2017, Koenig *et al.*, 1989, Le Rumeur, 2015, Robinson-Hamm and Gersbach, 2016). Deletions can occur anywhere within the dystrophin gene, but are often concentrated between exons 44 and 55 (Aartsma-Rus *et al.*, 2016). In-frame deletions in this region remove part of the rod domain (Blake *et al.*, 2002). The DMD cases resulting from point mutations and very small insertions/deletions primarily introduce premature stop codons (Blake *et al.*, 2002). These mutations seem to be evenly distributed throughout the gene (Blake *et al.*, 2002).

Dystrophin and the dystrophin-associated protein complex (DAPC)

The dystrophin-associate protein complex (DAPC) is present within muscle fibers. Some components are subsarcolemmal, some are associated with the cell membrane and some are immediately outside the sarcolemma. The DAPC provides mechanical support during contraction/relaxation and likely functions in signal transduction (Heydemann *et al.*, 2007). The DAPC is composed of dystrophin, cytoskeletal proteins, and proteins that form 3 separate complexes—the dystroglycan subcomplex, the sarcoglycan-sarcospan subcomplex and the cytoplasmic subcomplex (Ehmsen *et al.*, 2002, Sancar *et al.*, 2011). Proteins in these complexes include α- and β-dystroglycan, sarcoglycans, sarcospan, syntrophins, and dystrobrevin; abnormalities in these proteins may result in loss of membrane integrity, cell injury and myonecrosis (Kamdar and Garry, 2016, Quattrocelli *et al.*, 2017). The functions of these proteins have been reviewed in (Allen *et al.*, 2016). Mutations in some of the non-dystrophin DAPC-proteins or other glycosyltransferases that support O-mannosylation of α-dystroglycan cause non-DMD/BMD forms of muscular dystrophy (Guiraud *et al.*, 2015, Heydemann *et al.*, 2007).

Dystrophin is encoded by the *DMD* gene, which is the largest protein-coding gene in the human genome and is present on the X chromosome (Tuffery-Giraud *et al.*, 2017). The gene is ~2.5 Mb, composed of 79 exons, and results in a 427 kDa protein predominantly expressed in cardiac, smooth, and skeletal muscle (Nardes *et al.*, 2012, Sironi *et al.*, 2002). Interestingly, splice variants of dystrophin are also expressed in the brain (cerebral cortical, hippocampal, and Purkinje cell neurons neurons as well as glial cells), retina, and Schwann cells (Bies *et al.*, 1992, Sironi *et al.*, 2002). Altered dystrophin expression in these neurons is thought to explain the cognitive impairment often observed in DMD and BMD (D'Angelo *et al.*, 2011, Mazzone *et al.*, 2011). In muscle, dystrophin is a protein of low abundance (0.002% of skeletal muscle protein) located on the intracellular side of the sarcolemma (Brown *et al.*, 2012, Hoffman *et al.*, 1987). Functionally, dystrophin is thought to act as a molecular shock absorber, as part of the DAPC, by transmitting forces generated by contraction to the extracellular matrix (Guiraud *et al.*, 2015, Kamdar and Garry, 2016).

Utrophin, a dystrophin paralog

Utrophin is encoded by the *UTRN* gene on human chromosome 6 and mouse chromosome 10 (Helliwell *et al.*, 1992, Nguyen *et al.*, 1991, Schofield *et al.*, 1993). It is a 395 kDa protein expressed in skeletal, smooth, and cardiac muscle (Helliwell *et al.*, 1992, Pons *et al.*, 1994, Schofield *et al.*, 1993). Early in development, utrophin is present at the sarcolemma of skeletal muscle. It is progressively replaced by dystrophin, such that in adult muscle, utrophin is primarily expressed at myotendinous and neuromuscular junctions (Clerk *et al.*, 1993, Janghra *et al.*, 2016, Nguyen *et al.*, 1991, Schofield *et al.*, 1993, Tome *et al.*, 1994). Utrophin is also expressed in vascular smooth muscle and nerves (Helliwell *et al.*, 1992). In regenerating skeletal myofibers, utrophin is again expressed at the sarcolemma (Helliwell *et al.*, 1992). Structurally, utrophin shares 80% sequence homology with dystrophin (Schofield *et al.*, 1993, Tinsley *et al.*, 1992). Despite functional differences where dystrophin, but not utrophin, plays a role in microtubule organization and nNOS localization to the sarcolemma, several studies have shown that utrophin improves function in dystrophic muscle (Anthony *et al.*, 2014, Belanto *et al.*, 2014, Janghra *et al.*, 2016, Krag *et al.*, 2004, Rafael *et al.*, 1998, Squire *et al.*, 2002, Tinsley *et al.*, 1992).

Muscle pathology in DMD and BMD

Non-diseased skeletal muscle is composed of fibers that in cross section have a relatively uniform size and polygonal shape; there is very little endomysial connective tissue separating adjacent muscle fibers (Figure 1A) (Blake *et al.*, 2002). DMD muscle is characterized by variable degrees of atrophy, hypertrophy, myonecrosis, regeneration and endomysial fibrosis (Figure 1B) (Blake *et al.*, 2002, Mah *et al.*, 2016). The severity of histopathology ranges widely depending on the age of the patient and the muscle being evaluated. The absence of functional dystrophin prevents the connection between the intracellular cytoskeleton and the cell membrane, leading to repeated cycles of myonecrosis and regeneration associated with inflammation and repair (Allen *et al.*, 2016, Blake *et al.*, 2002, Falzarano *et al.*, 2015, Mah *et al.*, 2016, Zaharieva *et al.*, 2013). Within individual muscle fibers, the necrosis is segmental and regeneration relies upon satellite cell proliferation to produce myoblasts that eventually fuse to bridge the necrotic segment (Blake *et al.*, 2002). Macrophages are invariably present as they participate in phagocytosis of the

necrotic muscle fibers (Blake *et al.*, 2002, Madaro and Bouche, 2014). Lymphocyte infiltrates are variable, but only rarely are lymphocytes numerous enough to mimic myositis. Late in the disease process, myofibers are largely replaced by fibrofatty tissue (Blake *et al.*, 2002, Collins and Morgan, 2003).

In BMD, skeletal muscle pathology is similar to, though milder than that present in age-matched DMD patients (Kaido *et al.*, 1991, Tachi *et al.*, 1993). Interestingly, Kaido et al (1991) describe a distribution in BMD muscle pathology that segregates based on age at biopsy, not severity of disease presentation. In younger patients (15 years old), the pathology is predominantly active myonecrosis and regeneration, whereas, in older patients (>15 years old), chronic myopathic changes (increased internally-placed nuclei, split fibers, extremely hypertrophic muscle fibers, and endomysial fibrosis) were more likely to be observed (Ciciliot and Schiaffino, 2010, Kaido *et al.*, 1991, Tachi *et al.*, 1993). The entire spectrum of DMD and BMD histopathologic changes can also be observed in the various genotypes of congenital and limb-girdle muscular dystrophies (D'Angelo *et al.*, 2011, Robinson-Hamm and Gersbach, 2016).

Mechanism of injury in DMD

The mechanism of injury in DMD/BMD is multifactorial and complex. Early studies suggested that membrane damage and the consequences of Ca^{2+} influx were key to the pathology (Allen *et al.*, 2016, Blake *et al.*, 2002, Deconinck and Dan, 2007, Nowak and Davies, 2004). This was supported by EM evidence of focal disruptions in the cell membrane and elevations in intracellular Ca^{2+} concentration (Allen *et al.*, 2016). Later, studies in *mdx* mice, a mouse model that harbors a genetic mutation resulting in a DMD-like phenotype, demonstrated that muscle contraction is required for the initial muscle damage to occur (Allen *et al.*, 2016, Shoji *et al.*, 2015). In *mdx* mice with unlimited activity, histologic evidence of myonecrosis and regeneration first appears at 3–4 weeks of age and affects >50% of fibers by 8 weeks of age; whereas in muscle of limbs that were kept at a fixed length (short or long), evidence of damage (i.e. central nuclei) was not present (Allen *et al.*, 2016, Mokhtarian *et al.*, 1999). Similarly, in cultured *mdx* myotubes, resting intracellular Ca^{2+} levels were higher in spontaneously contracting myotubes versus those immobilized by tetrodotoxin (Allen *et al.*, 2016, Hopf *et al.*, 1996). Together, this suggests that dystrophin loss combined with calcium influx and muscle contraction results in muscle damage (Shoji *et al.*, 2015).

Additional studies demonstrate a role for loss of nNOS and nitric oxide (NO) and increases in reactive oxygen (ROS) and nitrogen species (RNS) in the pathology of DMD and BMD, though the true importance of nNOS loss is not known (see (Allen *et al.*, 2016) for detailed review). Neuronal nitric oxide synthase (nNOS), a signal transduction protein associated with the DAPC, produces NO, which may regulate local vascular perfusion, calcium mobilization, glucose metabolism and contractile function (Allen *et al.*, 2016, Guiraud *et al.*, 2015). In DMD patients, reduced nNOS activity results in impaired vasodilation and ischemia; however, some BMD patients lack the nNOS binding domain but remain mildly affected and ambulant, suggesting nNOS tethering at the sarcolemma is not an absolute requirement to have a mild phenotype (Anthony *et al.*, 2011, Guiraud *et al.*, 2015, Thomas,

2013, Torelli *et al.*, 2004). Loss of nNOS from the sarcolemma is also associated with an increase in the ROSproducing NADPH oxidase (NOX2) (Allen *et al.*, 2016). Increases in protein carbonyl groups and levels of isoprostanes, indicators of muscle oxidation and lipid peroxidation, respectively, have been demonstrated in muscle from *mdx* mice and DMD patients, suggesting a role for ROS in DMD pathology (Allen *et al.*, 2016, Grosso *et al.*, 2008, Hauser *et al.*, 1995, Haycock *et al.*, 1996, Messina *et al.*, 2006). Finally, studies in *mdx* mice suggest that stretch activation of NOX2 rapidly produces ROS, triggering Ca^{2+} entry through stretch-activated channels, leading to increased mitochondrial Ca^{2+} and ROS production, ultimately resulting in muscle damage and weakness (Allen *et al.*, 2016).

Experimental models of DMD

The number of experimental models for studying DMD is high, with more than 60 models available (McGreevy *et al.*, 2015). Stem cell models and non-mammalian models also have a role in understanding disease pathogenesis and effects of treatments on disease pathophysiology. While each has its limitations, each of the following model systems has provided significant information in understanding DMD.

Stem cell models

Although embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) with *dmd* gene mutations have been used to study the skeletal and cardiac muscle pathology and drug efficacy, the focus thus far has been on modeling cardiomayopathy (Kalra *et al.*, 2016, Smith *et al.*, 2016). Kalra et al (2016) suggest that cardiomyocytes derived from iPSCs provide a superior alternative to the *mdx* mouse to study the cardiac complications of DMD. First, *mdx* mice develop mild disease late in their lifespan, and *mdx* mice with a second knockout (double knock out (dko)) that develop cardiac disease earlier have a second gene mutation not present in humans (Kalra *et al.*, 2016). Second, mouse hearts differ physiologically from human hearts by having a faster heart rate (HR), a decrease in the force of contraction with increasing HR (versus an increase in force for humans), differences in ion channels that repolarize the plasma membrane, and differences in expression of α- vs β-myosin heavy chain (Kalra *et al.*, 2016). Third, iPSCs can be genetically modified using the Cas9/CRISPR (clustered regularly interspaced short palindromic repeat) system to correct the mutation (Kalra *et al.*, 2016). This provides an isogenic control in which the only difference between the two cell populations is the corrected gene sequence (Kalra *et al.*, 2016).

Non-mammalian models

The dystrophin gene is highly conserved, and homologues have been identified in vertebrates and invertebrates, including *Caenorhabditis elegans, Drosophila melanogaster*, and *Danio rerio* (Collins and Morgan, 2003, McGreevy *et al.*, 2015). While non-mammalian models have different musculature and pathology from their mammalian counterparts, their advantage is their physiologic simplicity and the ease of genetic manipulation (Collins and Morgan, 2003).

Zebrafish (*Danio rerio*) have rapid external development of translucent embryos that are easy to manipulate, which makes them attractive to researchers (Berger and Currie, 2012).

They have abundant skeletal muscle and express orthologues of most DAPC proteins, that have similar membrane localization as in the human (Collins and Morgan, 2003). Dystrophin-deficient zebrafish have been identified (*dmd*[ta222a]) and created by morpholino knockdown, and their histologic features include extensive muscle degeneration/necrosis, fibrosis, inflammation, activation of muscle stem cells and variation in fiber diameter (Berger *et al.*, 2010, Berger and Currie, 2012). Similar to humans, zebrafish express dystrophin in embryos first at a junctional myotendinous location in the embryo and later at non-junctional sarcolemma (Berger *et al.*, 2010). Failure of this transition is believed to be the major cause of the human pathology (Berger *et al.*, 2010, Berger and Currie, 2012). Dystrophin-deficient zebrafish have been used to study exon-skipping therapies, and while they demonstrate that 20–30% of normal dystrophin levels are needed to rescue a severe phenotype in this context, they cannot be used to study issues related to systemic antisense oligonucleotide administration (Berger *et al.*, 2011, Berger and Currie, 2012).

C. elegans are readily genetically manipulatable, and can be grown *en masse*, making them ideal for high-throughput genetic and pharmacologic studies (Collins and Morgan, 2003). They express the dystrophin homologue *dys-1*, and when in a mutated *MyoD* genetic background, *dys-1* mutations lead to progressive impairment of locomotion (Collins and Morgan, 2003, Gaud *et al.*, 2004). While affected nematodes have widespread degeneration of the body wall muscles, their muscular structure is dramatically different from that of mammals (Gaud *et al.*, 2004). Their muscle fibers do not fuse and are incapable of regeneration (Gaud *et al.*, 2004). Dystrophin-deficient *C. elegans* have been used to screen numerous compounds covering a wide spectrum of targets (Gaud *et al.*, 2004). In these nematodes, prednisone reduces the number of degenerating fibers by 40%. Since they do not mount an inflammatory response to myonecrosis, this suggests that prednisone exerts a direct effect on muscle survival (Gaud *et al.*, 2004). *C. elegans* has also been used to study Ca^{2+} signaling through their voltage-dependent Ca^{2+} channel (VDCC) (Zhan *et al.*, 2014). In these studies, dystrophin was shown to be a load-bearing scaffold protein that influences membrane stiffness and transduces muscle tension to VDCCs (Zhan *et al.*, 2014).

Drosophila melanogaster is useful because of its short generation time, large number of progeny, simple morphology, and segmental body plan (Kreipke *et al.*, 2017, Plantie *et al.*, 2015). The *Drosophila* DAPC also contains the major components present in the mammalian DAPC (Kreipke *et al.*, 2017). *Drosophila* are useful for studying degenerative muscle diseases because their muscles lack satellite cells responsible for regeneration, allowing differentiation between phenotypes stemming directly from degeneration vs. those arising from failure of regeneration (Kreipke *et al.*, 2017). Similar to *C. elegans*, *Drosophila* has also been used for small molecule therapeutics (Pantoja and Ruohola-Baker, 2013).

Rodent models

The *mdx* mouse is commonly used to study DMD and the dystrophic phenotype arises because of a point mutation (C to T transition) in exon 23, which results in a stop codon and truncated protein (De Luca, 2012, McGreevy *et al.*, 2015, Selsby *et al.*, 2015). While the *mdx* mouse is well-characterized (Table I), the disease phenotype in all commonly examined muscles except for the diaphragm is much milder than the human DMD phenotype (Selsby

et al., 2015). These mice exhibit only a 25% decrease in lifespan (vs 75% decrease in humans) and display minimal clinical signs (McGreevy *et al.*, 2015). Double knockout mice (dko) have been developed with varying effects on phenotype (McGreevy *et al.*, 2015, Selsby *et al.*, 2015). For example, dko mice lacking dystrophin and utrophin have a severe DMD-like phenotype with a significantly shortened lifespan (Deconinck *et al.*, 1997). Second mutations have been introduced to reduce compensatory mechanisms (i.e. loss of utrophin), humanize mice (i.e. inactivation of cytidine monophosphate sialic acid hydrolase (Cmah)), reduce muscle regeneration (i.e. elimination of MyoD), and to mutate genes involved in cytoskeleton-ECM interactions (i.e. desmin and laminin); however, this introduces a second mutation not present in human DMD, complicating data interpretation (Deconinck *et al.*, 1997, McGreevy *et al.*, 2015, Selsby *et al.*, 2015). Finally, *mdx* mice with second mutations to reduce inflammation have been developed (McGreevy *et al.*, 2015). While mice are useful for studying pathogenesis, they are poor models of responses to gene therapy vectors. In mice, IM injection results in persistent adeno-associated virus (AAV) transduction (compared with nominal transduction in humans) (McGreevy *et al.*, 2015).

Two different strains of dystrophin-deficient rats have been created and characterized (Larcher *et al.*, 2014, Nakamura *et al.*, 2014). These rats have deletions either between exons 3 and 16, or in exon 23 (Larcher *et al.*, 2014, Nakamura *et al.*, 2014). They have undetectable levels of dystrophin (by western blot) and <5% dystrophin positive fibers by IHC (Larcher *et al.*, 2014, Nakamura *et al.*, 2014). Rats with mutations in exon 23 display progressive myonecrosis and regeneration along with fibrofatty replacement (Larcher *et al.*, 2014). Significant fibrosis arises within the heart, and they develop dilated cardiomyopathy as indicated by eccentric hypertrophy and diastolic dysfunction (Camacho *et al.*, 2016). Because of the cardiac similarities of these rats with DMD patients, they are considered to be a better model than *mdx* mice for studying DMD cardiomyopathy (Camacho *et al.*, 2016, Larcher *et al.*, 2014).

Large animal models of DMD

DMD models have been established in the cat, dog, and pig (see Table II) (Klymiuk *et al.*, 2013, Kornegay, 2017, McGreevy *et al.*, 2015). Dystrophin gene disruption has been produced in a rhesus monkey, however a model has not been established (Chen *et al.*, 2015). These models are useful for investigating responses to viral vectors. Canine models have similar nominal transduction rates as humans, and there is robust immune rejection following gene therapy as in DMD patients (Duan, 2015). These models are also useful for identifying issues from large-scale vector production and delivery (Duan, 2015). While some of these models more closely replicate the human disease than the non-mammalian and rodent models, they are costly and availability for research is limited. Additionally, the golden retriever muscular dystrophy dog (GRMD) and pig models have breeding issues. Finally, each of these large animal models is based on a single *DMD* mutation, which cannot model the heterogeneity of human mutations (McGreevy *et al.*, 2015).

In the dog, dystrophin deficiency has been documented in 20 different dog breeds and colonies of affected animals have been established for 7 breeds (McGreevy *et al.*, 2015). The GRMD is the most widely used dog model; these dogs have a point mutation in intron 6, one

of the hotspots of mutation in DMD patients (McGreevy *et al.*, 2015). This mutation results in exclusion of exon 7 and a frame shift that introduces a stop codon in exon 8 (Nakamura and Takeda, 2011, Selsby *et al.*, 2015). The GRMD dogs display a similar disease course to that of DMD patients, with limb weakness and exercise intolerance at 2–3 months of age, muscle atrophy, dysphagia, abnormal weight gain, signs of heart disease by 6 months, and death at about 3 years (McGreevy *et al.*, 2015, Nakamura and Takeda, 2011). Unfortunately, these dogs have a "honeymoon" period around 6–10 months, where disease progression stabilizes and the GRMD and human DMD phenotypes diverge (Kornegay, 2017, McGreevy *et al.*, 2015). Though the genotype of these dogs remains consistent, phenotype can be variable and is likely due to genetic modifiers, including secreted phosphoprotein 1 (SPP1) and latent transforming growth factor β binding protein 4 (LTBP4) (Klymiuk *et al.*, 2016, Kornegay, 2017). In the Cavalier King Charles spaniel (CKCS) with muscular dystrophy, the missense mutation was identified in exon 50, another hotspot for mutations in DMD patients, and these dogs are valuable for studying exon-skipping drugs (Nakamura and Takeda, 2011, Walmsley *et al.*, 2010). Because of the genetic limitations of this breed, Professor Piercy at the Royal Veterinary College (United Kingdom) has engineered a model of DMD in the beagle with this same mutation, and the natural history study of these dogs is ongoing (Porter, 2015).

Dystrophin-deficiency with multifocal myonecrosis and regeneration has been identified in cats, and the large deletion mutations are located at the muscle and Purkinje promoters of the dystrophin gene (Carpenter *et al.*, 1989, Gaschen and Burgunder, 2001, Klymiuk *et al.*, 2016, Selsby *et al.*, 2015). Affected cats develop hypertrophy of the tongue, neck and shoulder muscles, as well as megaesophagus, hepatosplenomegaly, and kidney failure (Nakamura and Takeda, 2011). In contrast to DMD patients, they develop hypertrophic cardiomyopathy (Gaschen *et al.*, 1999, McGreevy *et al.*, 2015). With the difference in skeletal and cardiac muscle phenotype as well as tongue and diaphragm hypertrophy leading to feeding difficulties and death, these cats are rarely used as a research animal (Nakamura and Takeda, 2011, Selsby *et al.*, 2015).

Dystrophin-deficient pigs have been generated by deleting exon 52 (Klymiuk *et al.*, 2013). These pigs demonstrate similar progressive multifocal myonecrosis and regeneration as in DMD patients (Klymiuk *et al.*, 2013, Klymiuk *et al.*, 2016). Interstitial fibrosis and mononuclear cellular infiltration is also present (Koenig *et al.*, 1989, Le Rumeur, 2015). These pigs also demonstrate similar functional impairment, but in an accelerated manner. Similar to the CKCS and beagle models of DMD with deletions of exon 50–52, the pig model can be used to study exon-skipping drugs (Klymiuk *et al.*, 2016). The pigs can also be used to study proteomic changes at the various disease stages (Frohlich *et al.*, 2016). Affected pigs have a maximum life expectancy of 14 weeks and since the male pigs do not live to breeding age, these pigs cannot be propagated by breeding (Klymiuk *et al.*, 2016). To overcome these breeding difficulties, female carriers have been generated and have been mated with wild type boars. Studies characterizing the offspring are in progress (Klymiuk *et al.*, 2016).

Evaluating efficacy of therapeutic candidates

DMD pipeline

There currently is no cure for DMD/BMD and only two therapies are approved for use in these patients in the United States. Additional therapeutics are approved in Europe. One of the approved US therapies reduces inflammation (Emflaza™, the glucocorticoid deflazacort). Inflammation plays an important role in the pathogenesis of DMD. Early inflammation removes dead myofibers; however, without an increase in dystrophin, which is associated with a reduction in inflammatory marker expression, a chronic inflammatory state is established with overexpression of NF-kB, TNF-alpha and dysregulation of M1/M2 macrophages (De Luca, 2012, Madaro and Bouche, 2014). Glucocorticoids increase muscle strength, prolong independent ambulation, delay onset of cardiomyopathy, improve pulmonary function and reduce the incidence of scoliosis (Griggs *et al.*, 2016). While clinical improvement is observed with these therapies, the improvement is temporary (disease progression is delayed), and treatment is associated with side effects, including weight gain, cataracts and bone fractures (Lim *et al.*, 2017). Deflazacort is associated with less weight gain than prednisone (which is used off-label), and this helps reduce mechanical load on impaired muscles (Griggs *et al.*, 2016). Because of the lack of long-term efficacy of glucocorticoids, potentially more curative therapies for DMD are in development. Strategies include cell-based strategies (transplantation of healthy myoblasts or stem cells into patients), gene-based strategies (gene therapy, exon-skipping drugs), utrophin modulators, and calcium modulators (Allen *et al.*, 2016, Willcocks *et al.*, 2016). Eteplirsen (Exondys 51™), the second US-approved drug, promotes dystrophin production by restoring the translational reading frame of the DMD gene by skipping translation of exon 51 (Lim *et al.*, 2017). Ataluren (Translarna™), approved for use in the European Union, is used in patients with DMD caused by a nonsense mutation (nmDMD) (Bushby *et al.*, 2014). Ataluren is believed to interact with ribosomes to read through premature stop codons, allowing production of the full-length protein (Welch *et al.*, 2007).

End-points used for assessing clinical improvement

Methods for measuring efficacy can be broadly classified into two categories—clinically meaningful and surrogate. Clinically meaningful endpoints relate to quality of life, and in DMD patients, include the 6-minute walk test (6MWT), timed function tests (time to stand from a supine position, time to walk/run 10 m, time to climb or descend 4 standard-sized stairs) and quantitative strength using hand-held myometry (McDonald *et al.*, 2013b). Surrogate endpoints are used to predict clinical benefit and include muscle enzymes, forced vital capacity and imaging (MRI-T2/H[1]-MRS, echocardiography), quantitative assessment of dystrophin in biopsies, and gene expression profiling. The 6MWT, MRI-T2/H[1]-MRS, and quantitative assessment of dystrophin in biopsies will be discussed below.

The 6MWT is an indicator of global muscle and cardiorespiratory function (Allen *et al.*, 2016). In this test, boys walk down a flat, straight, well-lit, seldom-traveled corridor (see Figure 2) (McDonald *et al.*, 2010, McDonald *et al.*, 2013a). A tape line indicates the length of the course and is marked in 1m increments, and cones are used at the beginning and end of the course (McDonald *et al.*, 2010, McDonald *et al.*, 2013a). Distance traveled at each

minute and location of falls are recorded (McDonald *et al.*, 2010, McDonald *et al.*, 2013a). Testing staff can provide verbal encouragement to maintain attention to the task, and "safety chasers" can help fallen boys stand (McDonald *et al.*, 2013a). While the 6MWT is becoming the gold standard for assessing therapies, it does have practical difficulties (Allen *et al.*, 2016). In normal boys, the distance traveled increases with age, but decreases as the disease becomes more debilitating (Allen *et al.*, 2016). Thus, the test is insensitive to disease progression in younger boys. Also, distance increases in a drug trial are only unequivocally the result of the drug if the disease is in a stage of decline (Allen *et al.*, 2016). This complicates data interpretation in younger boys, who might benefit most from the therapy under study.

Magnetic resonance imaging (MRI) and spectroscopy (MRS) are promising surrogate endpoints for DMD clinical trials (Willcocks *et al.*, 2016). MRI-T2 images allow assessment of muscle damage, inflammation and fat infiltration, and ^{1}H-MRS allows assessment of fat infiltration into muscle (i.e. fat fraction) (Willcocks *et al.*, 2016). A study of 109 boys (ages 5.0–12.9 years old) demonstrated an increase in MRI-T2 signal and ^{1}H-MRS fat fraction even in boys with stable or an improved 6-minute walk distance, suggesting that these MR biomarkers can detect subclinical disease progression (Willcocks *et al.*, 2016).

While this technique requires trained personnel and careful calibration across test sites, it allows inclusion of younger test subjects (Willcocks *et al.*, 2016). Additionally, these techniques allow for serial assessment of multiple muscles without needing multiple invasive muscle biopsies.

Dystrophin expression (protein and transcript) can be measured in excised muscle specimens with a variety of techniques, including western blot, mass spectrometry, ELISA, RT-qPCR and IHC/IF with scoring (Anthony *et al.*, 2012, Anthony *et al.*, 2014, Brown *et al.*, 2012, Nguyen *et al.*, 1990, Nicholson *et al.*, 1990). Currently, quantifying dystrophin in muscle is the earliest indicator of efficacy; however, accurate assessment of dystrophin expression is challenging because of the low abundance and large size of the protein (Anthony *et al.*, 2012, Merlini and Sabatelli, 2015, Taylor *et al.*, 2012). Western blot, mass spectrometry, and ELISA excel at quantifying dystrophin in whole tissue samples, but lack fiber-by-fiber data and some techniques are challenged by limits of quantification (i.e. LLOQ of 3–5% for mass spectrometry is too high for DMD patients) (Anthony *et al.*, 2014, Brown *et al.*, 2012). While RT-qPCR does not measure dystrophin levels, this method can be used to measure dystrophin mRNA levels in patients treated with exon-skipping drugs (Anthony *et al.*, 2012). IHC/IF excels at providing fiber-by-fiber data, exclusive assessment of muscle fibers (allowing exclusion of areas of necrosis, fibrofatty infiltrate), assessment of revertant fibers, measurement of expression heterogeneity across the tissue and within individual muscle fibers (completeness of membrane expression), and measurement of variation in staining intensity. Manual methods can provide robust data, but they must be carefully controlled or the quantification of dystrophin is likely to be affected by scoring biases (Aeffner *et al.*, 2017, Arechavala-Gomeza *et al.*, 2010b, Beekman *et al.*, 2014). Despite these challenges, there is strong correlation of the number of positive fibers counted in IF-stained sections and protein quantified by western blot, suggesting that IF/IHC and western blot are comparable methods (Nicholson *et al.*, 1993). Finally, care needs to be taken when selecting an antibody-

based detection method, as inaccurate dystrophin levels will be reported if the antibody selected binds an epitope lost by a patient's specific mutation.

Image analysis in non-clinical and clinical studies

Image analysis methods quantify protein expression in IF or IHC images. Methods range from scoring small, high power fields (HPF) manually to scoring whole-slide images manually to scoring whole-slide images with image-analysis software. While HPFs are easier to score, this method is subject to bias, particularly in image selection. Scoring whole-slide images prevents the sampling bias, but manual evaluation can be time-consuming. Digital image analysis of whole sections is becoming the preferred method (Janghra *et al.*, 2016). In addition to sampling bias, scoring biases can exist with manual scoring of either HPFs or whole-slide images. Unless scorers and scoring conditions are carefully controlled, scorers are prone to drift in threshold and estimating the percentage of membrane with positive staining, both resulting in an increase in number of positive fibers over time. The visual trap in Figure 3 demonstrates how context can impact perceived fiber intensity and why it is necessary to constantly reassess the performance of manual scorers. In this example, the shadow cast by the cylinder obscures the true intensity of the adjacent squares.

Several efforts have been made to objectively measure dystrophin intensity in DMD muscle biopsies. In the first two reports, sections were labeled with dystrophin only, circles were placed over select areas of the sarcolemma, and maximum membrane intensity within that circle was calculated (Arechavala- Gomeza *et al.*, 2010a, Arechavala-Gomeza *et al.*, 2010b). In the third report, sections were dual labeled with dystrophin and spectrin (a cytoskeletal protein lining the inner surface of the plasma membrane), and the average dystrophin intensity from all muscle fibers was calculated (Taylor *et al.*, 2012). In the fourth report, sections were dual-labeled with dystrophin and spectrin, and the distribution of dystrophin intensity for each fiber was calculated (Beekman *et al.*, 2014). Together, these methods present pros and cons of measuring dystrophin in IF-stained sections. Amongst the four reports, methods differed in measuring intensity in both positive and negative fibers, measuring mean vs. maximum intensity vs. distribution of intensity, and measuring intensity in a portion of the membrane vs. the entire membrane. While these early attempts provide reproducible data, they lack the sophistication of newer methods.

Considering the positives and negatives of the current solutions, our group has developed a software solution, MuscleMap™ that assesses dystrophin or utrophin intensity in individual fibers identified by a membrane-associated marker (i.e. laminin-α2 or spectrin) in a duplex immunostaining assay (manuscript submitted; Figure 4). Images are captured under identical conditions with a scanner qualified for reproducible image capture. MuscleMap identifies all muscle fibers using the membrane-associated marker, and then identifies and calculates the amount of dystrophin or utrophin. MuscleMap data outputs include membrane completeness (a measure of the circumference of the membrane with dystrophin or utrophin expression), biomarker intensity (which can be gated by thresholds into positive/negative or 0–3+ bins (Figure 5)), and a mathematic combination of these two values (the 4CC score). MuscleMap also calculates morphometric features (such as minimum fiber diameter), assesses levels of intra- and inter-fiber heterogeneity, and counts the number of revertant fibers. While first

developed to analyze IF-stained images, MuscleMap can now be used to analyze IHC-stained images, as referenced in the future steps below and illustrated in Figure 6 (Tinsley *et al.*, 2016). Overall, these image analysis techniques on images captured under controlled conditions help to overcome biases in sample selection as well as manual scoring. These standardized methods can be used to accurately and consistently measure biomarker changes to evaluate drug efficacy.

Future directions

While the number of potential therapies is increasing, <20% of Phase II trials are successful (Kornegay, 2017). These clinical trials often fail from either safety concerns or lack of efficacy, which results from the inability to accrue sufficient patients to achieve the necessary power (Kornegay, 2017). Because of the challenges in recruiting large numbers of patients, classical large clinical trial designs are not practical or even possible. This raises the question: are we using the best primary endpoint for these trials? Do we need to refine the methods of the 6MWT/timed functional tests or do we need to choose an alternative? In late 2016, the United States Congress passed the 21st Century Cures Act, which allows companies to seek accelerated approval using surrogate biomarkers that are likely to predict clinical benefit. One proposed alternative is using MR-based tools (i.e. MRI-T2 or ^{1}H-MRS) to measure fat fraction. As part of their assessment of MRI/MRS techniques, Willcocks et al (2016) simulated patient numbers needed to detect stabilization of disease progression with 80% power. Their simulation suggests that only 13 patients/group would be required with this surrogate endpoint (Willcocks *et al.*, 2016).

Similarly, tissue collection techniques and muscle based measurements of dystrophin may benefit from refinement. Currently, open biopsies are used to collect muscle specimens (Kinali *et al.*, 2011). The advantages of open biopsy are direct visualization, a relatively large quantity of muscle obtained, and ease of freezing muscle in the correct orientation for IF/IHC studies. Needle biopsies might provide an alternative method of collection. The smaller incision size might make it more palatable for biopsies to be taken from multiple sites, allowing assessment of heterogeneity of disease. The downside to the small sample size obtained is that it may be insufficient for some of the in vitro techniques used, particularly IF/IHC. Needle biopsy sample size may obscure fiber orientation, which is particularly important for assessing dystrophin or other biomarker expression using IF/IHC-based assays.

Additionally, staining is darker at the edge of samples for some antibodies ("edge effect"), and this edge effect may hinder interpretation in small samples. Imprecise assay performance can result in under- or over-estimation of protein levels. Alternatively, transitioning IF assays into IHC assays that utilize more robust visualization components may be beneficial. While fluorescent detection allows for easier multiplexing signal separation, better target co-localization, and higher dynamic range, IF-stained slides have lower sensitivity and are susceptible to photobleaching. By contrast, IHC has greater sensitivity and a longer lasting signal, which may prove beneficial during clinical trials. Our lab has developed IHC methods for measuring utrophin/laminin-α2 and developmental

myosin heavy chain (Figures 6A, 6B, respectively) and image analysis can reliably separate those signals to facilitate accurate quantification of multiplex assays (Tinsley *et al.*, 2016).

Acknowledgments

Abbreviations

AAV	Adeno-associated virus
BMD	Becker Muscular Dystrophy
CV	Coefficient of variation
DMD	Duchenne Muscular Dystrophy
ELISA	Enzyme-Linked Immunosorbant Assay
EM	Electron microscopy
FITC	Fluorescein Isothiocyanate
IF	Immunofluorescence
IHC	Immunohistochemistry
IM	Intramuscular
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
TRITC	Tetramethylrhodamine

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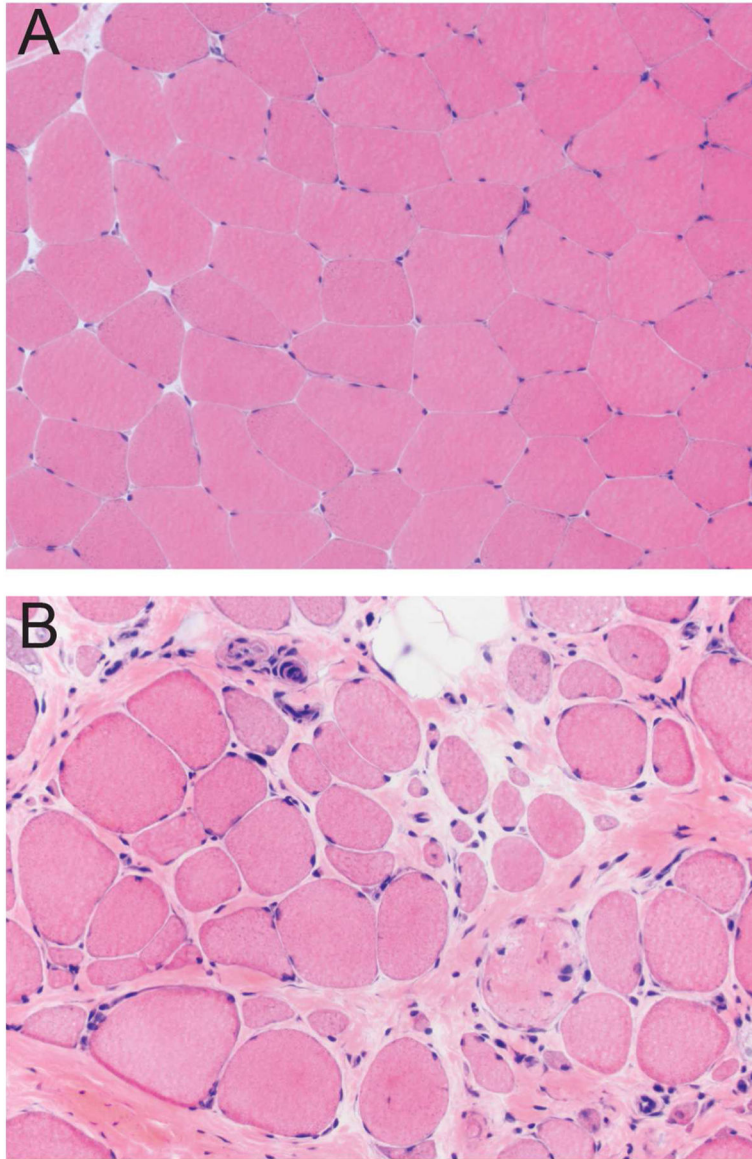
Figure 1. Histologic lesions of DMD muscle

The section of normal muscle (A) demonstrates myofibers that are uniform in size, polygonal in shape, and evenly spaced with very little endomysial connective tissue. The section of DMD muscle (B) shows increased myofiber size variation (atrophy and hypertrophy), internal nuclei, myonecrosis, endomysial fibrosis, and fatty replacement. Hematoxylin and eosin.
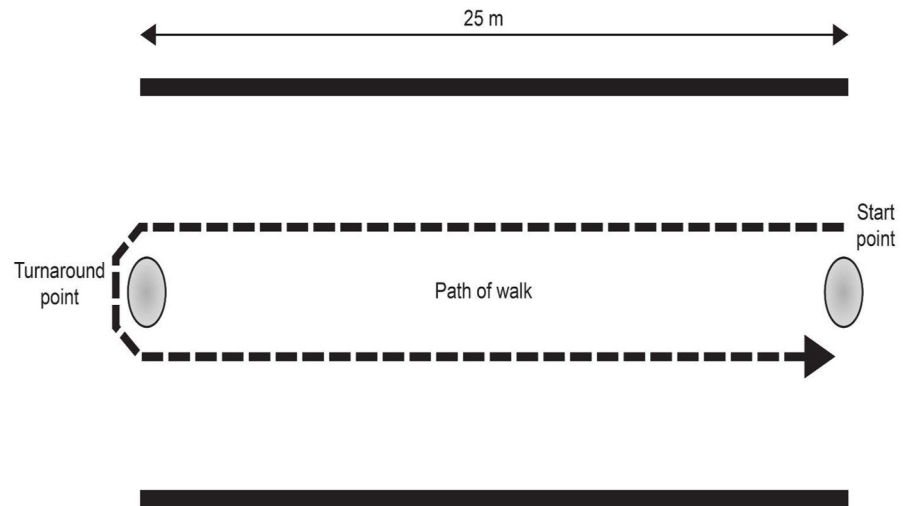
Figure 2. Representative course for the 6-minute walk test
This image shows the course for the 6-minute walk test (6MWT). A strip of tape 25 m long is placed in the center of a quiet, well-lit corridor at least 8 feet wide. Cones are positioned at the beginning and end of the course. Distance traveled at each minute and total are measured along with locations of falls.
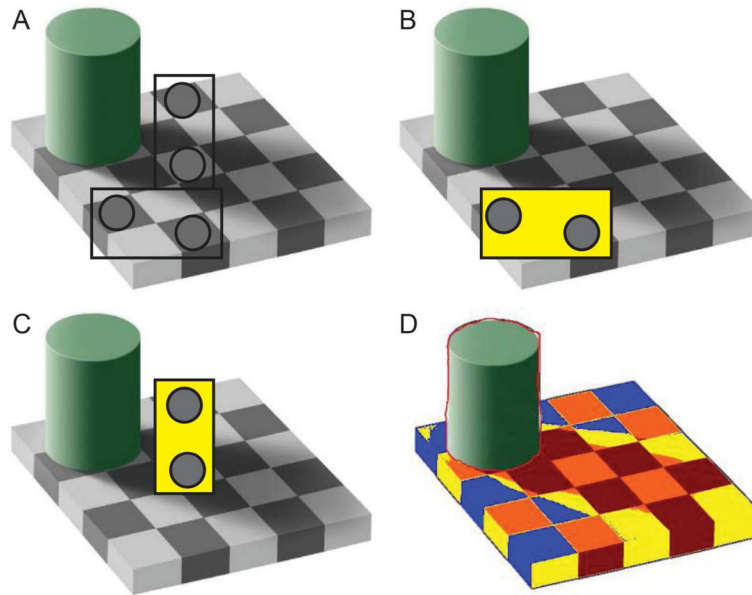
**Figure 3. Adelson's visual trap**

In this cognitive trap, two pairs of boxes are compared without (A) and with masks (B, C) to demonstrate how the brain is tricked into seeing boxes of equal intensity as different intensity (C). The image in D displays an image-analysis markup where boxes have been colored by intensity (i.e. all red boxes are the same intensity, etc.). Adapted from Adelson, EH, Lightness Perception and Lightness Illusions. In *The New Cognitive Neurosciences*, 2nd Edition. MIT Press (2001)
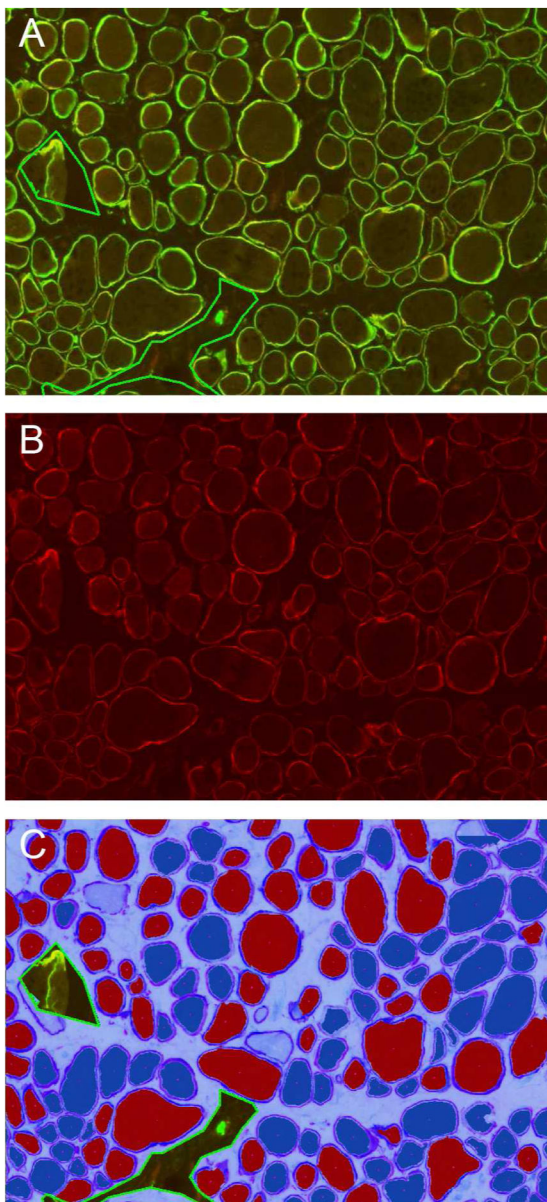
**Figure 4. MuscleMap consistently scores protein expression in muscle fibers**
This panel shows the dual labeled assay in A (TRITC-dystrophin, FITC-spectrin), with just the TRITC-dystrophin image in B and the image analysis markup in C showing positive and negative fibers. A fiber is classified as negative (dark blue cytoplasm in C) or positive (red cytoplasm in C) based on achieving a minimum with respect to membrane intensity and % circumference at that threshold. The image in C shows three areas of artifact excluded from analysis (indicated by the green line) as well as several fibers not meeting the fiber definition (light blue center) and not included in the analysis.
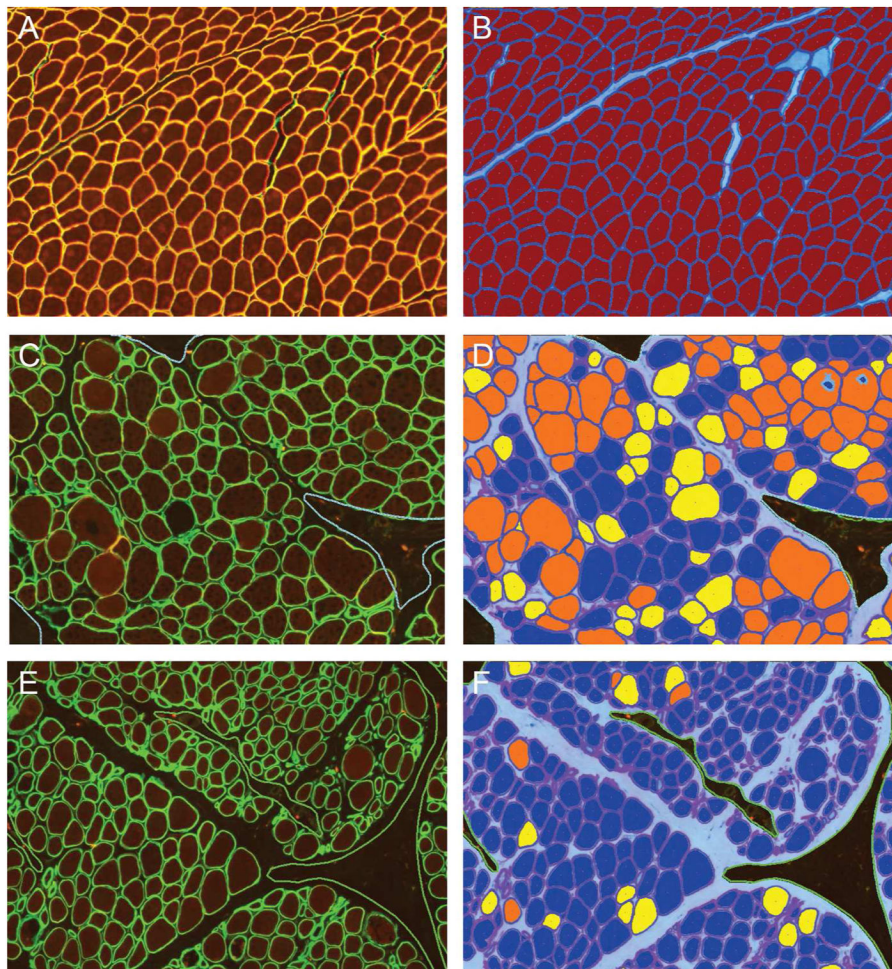
Figure 5. MuscleMap separates muscle fibers by expression intensity

This panel shows the dual labeled images in A, C, and E and the respective image analysis markups in B, D and F. In B, D and F, muscle fibers have been classified by intensity, where dark blue = negative, yellow = low intensity, orange = medium intensity and red = high intensity. Similar to Figure 4, areas of artifact (dashed green line) and myofibers not meeting the fiber definition (light blue center) were not included in the analysis. Muscle in A, C and E are from a non-DMD/non-BMD control, BMD patient and DMD patient, respectively.
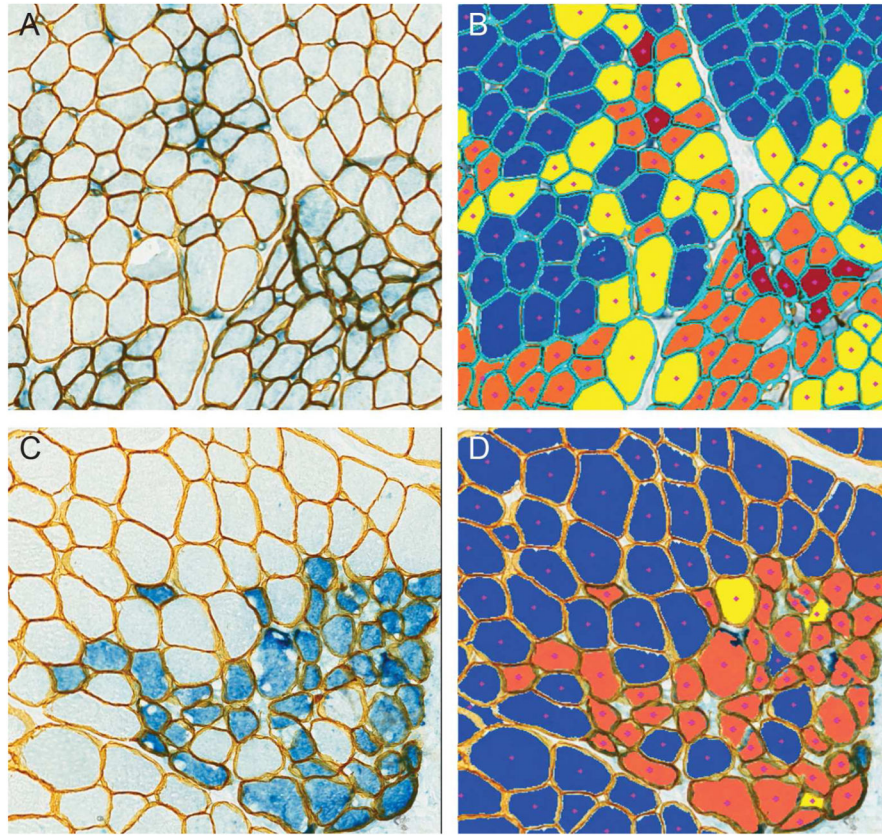
Figure 6. MuscleMap measures pathologist-impossible endpoints

This panel shows DMD muscle samples stained with two duplex IHC assays: in A, utrophin is in blue and laminin-α2 is in yellow, and in C, developmental myosin heavy chain is in blue and laminin-α2 is in yellow. In images B and D, muscle fibers have been classified by intensity, where dark blue = negative, yellow = low intensity, orange = medium intensity and red = high intensity. Myofibers not meeting the fiber definition (uncolored) were not included in the analysis.

Table I

Comparison of clinical manifestations and histopathology of dystrophin-null mice, dogs and humans

Feature	Humans	Mice	Dogs
Clinical manifestations			
Birth body weight	Normal	Normal	Normal
Adult body weight	< Normal	Normal	< Normal
Clinical course	Severe, progressive	Mild, non-progressive	Severe, progressive
Lifespan	25% of normal	75% of normal	25% of normal
Neonatal death	Rare	Rare	~25% of affected dogs
Age at first clinical signs	2–4 years old	15 months	Birth to 3 months
Loss of ambulation	Common at early teenage	Rare	Uncommon
Muscle wasting	Progressive	Minimal until 15 months	Progressive
ECG abnormalities	Frequent	Frequent	Frequent
Cardiomyopathy	Evident at 16 years	20 months; dilated (female) and hypertrophic (male)	Detectable at 6 months by echocardiography
Cognitive and CNS defects	1/3 of affected individuals	Mild	No information available
Histopathology			
At birth	Minimal	Minimal	Minimal
Acute necrosis	None	2–6 weeks	None
Limb muscle fibrosis	Extensive and progressive	Minimal in adult	Extensive and progressive
Muscle regeneration	Poor	Robust	Poor

Adapted from (McGreevy *et al.*, 2015). Shading indicates characteristics that are similar to human disease.

Table II

Animal models of DMD

Species	Comments
Non-mammalian	*C. elegans*, *Drosophila*, Zebrafish; rarely used in gene therapy studies
Mouse	Dystrophin-deficient mice; 17 models; most have point mutations, some have deletions or insertions
Mouse	Immune deficient mdx mice; 4 models; differing combinations of immune cell and cytokine deficiency; can be used to study cell or gene therapy without the compounding effects of the host immune response
Mouse	Phenotypic dko mice; 13 models; all result in severe dystrophic phenotype
Mouse	Dko mice with phenotype similar to mdx; 3 models
Mouse	Dko mice with reduced disease; 11 models
Mouse	Transgenic mdx mice; 13 models
Dog	22 breeds; most are case reports with no colony established; 7 have established colonies
Rat	2 models; exon deletion
Cat	2 types; exon deletion; spontaneous mutation; rarely used in gene therapy studies
Pig	1 BMD = missense mutation, 2 DMD = deletions of exon 52

Adapted from (McGreevy *et al.*, 2015).