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First, do no harm: the role of preclinical animal models in predicting adverse events in gene therapy clinical trials for Duchenne muscular dystrophy and X-Linked myotubular myopathy

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Abstract

The occurrence of severe adverse events (SAEs) in patients with Duchenne muscular dystrophy (DMD), X-linked myotubular myopathy (XLMTM), and other neuromuscular diseases treated with adeno-associated virus (AAV) constructs has prompted studies to improve the safety and efficacy of gene therapy. Physicians have weighed the medical tenet of "first, do no harm" against the perspective of patients with progressive life-threatening conditions who may accept greater risk. Regarding SAE pathogenesis, discussion has focused on total AAV exposure and patient mutations more likely to induce immunity, while stressing the limitations of animal models in predicting adverse events. Therapeutic strategies for reducing side effects have employed more myotropic AAV serotypes and efficient transgenes. Other recommendations include excluding certain *DMD* gene mutations associated with SAEs and substituting less immunogenic transgenes such as utrophin (DMD) and myotubularin-related protein (XLMTM). For the sake of preclinical studies, emphasis has been placed on outbred rodents and larger animals that better predict immunity. Here, the absence of side effects in canine DMD and XLMTM models might be explained

partly by phenotypic differences between affected humans and dogs. Specifically, dystrophin- and myotubularin-deficient dogs exhibit milder lesions, including less muscle fat deposition and the absence of hepatopathy, respectively, which could lead to reduced immune responses to AAV constructs. To better predict future problems, thought should be given to tracking early subclinical markers of the innate immune response, especially complement activation. Regardless of steps taken to improve the predictive value of animal models for SAEs, some questions will only be answered through human clinical trials after carefully considering the risk-benefit ratio.

Keywords: Duchenne muscular dystrophy, X-linked myotubular myopathy, animal models, severe adverse events (SAEs)

INTRODUCTION

The long-held promise of gene therapy to improve the lives of individuals with devastating diseases is finally being realized, with over 2,700 studies currently listed on the ClinicalTrials.gov website. This includes a variety of gene therapy products, like plasmid DNA, gene editing technology, viral vectors, and stem cells. Among viral vectors, adeno-associated viruses (AAVs), adenoviruses, and lentiviruses are used most, with AAVs predominating[1]. The original appeal of AAVs was based on their ability to achieve widespread gene transfer at clinically relevant doses in mice, dogs, and nonhuman primates (NHPs)[2]. However, severe adverse events (SAEs), not predicted by preclinical studies, have recently been seen in Duchenne muscular dystrophy (DMD) and X-linked myotubular myopathy (XLMTM) patients after AAV-transgene therapy[2-8]. Similar SAEs have been reported with AAV therapy in spinal muscular atrophy[5-9], as well as Pompe[5-9] and Danon[5] glycogen storage diseases. Taken together, this has raised questions about the ability of canine and other animal models to predict SAEs and suggests that alternative strategies may be needed.

HIPPOCRATIC OATH AND ETHICAL CONSIDERATIONS

The ethical standard "first (above all), do no harm," from the Latin *primum non nocere*, has guided physicians in treating patients since antiquity[10,11]. Usually attributed to the Greek physician Hippocrates and associated with the Oath bearing his name, the phrase's exact origins and meaning are controversial due to nuances of translation, shortcomings of historical accounts, and the changing moral values of society[12,13]. While the precise wording, "first, do no harm," is not included in the original Hippocratic Oath, the phrase can be inferred from other wording and additional writings by Hippocrates and his students during this period[11].

Regardless of the exact phrase used and to whom the expression is attributed, a distinction must be made between the potential for side effects of any treatment and those caused by negligence (so-called injustice or *non-maleficence*)[11]. This is especially relevant with devastating diseases for which a higher risk-benefit ratio could logically be tolerated. In this context, the Oath is seen by some as paternalistic without sufficient regard given to the perspective and autonomy of patients and their parents[10,14]. Indeed, surveys show that Duchenne patients may be more willing to accept a higher risk of SAEs than their treating clinicians[6]. This is an example of "shared decision making" employed in cancer treatment[15], wherein patients accept greater risks while physicians focus more on the possibility of adverse events[16].

Finally, from an ethical standpoint, there are concerns regarding the limited access of older, non-ambulatory DMD patients to any impactful treatment, especially gene therapy, despite potential benefits for upper limb and cardiopulmonary function[17,18]. This situation is troubling because both the relative risks and benefits of genetic treatments are typically worsened by advancing age. To ensure that the needs of these boys and young men with DMD are adequately addressed, it is essential to continuously update

outcome information for patients, families, and physicians. One approach could involve creating a database of anonymized patient ages, interventions, and outcomes, initially available to physicians at specialty centers of excellence, where the highest-risk, oldest patients are most likely to seek evaluation for treatment. Only with access to this granular information can physicians maintain the standard of *primun non nocere* in the era of extended indications for complex biologics to treat lethal diseases (see the FDA's recent approval of Sarepta's microdystrophin Elevidys for non-ambulatory patients below).

The experiences of all five authors have shaped this review. Stedman and Byrne addressed the ethical principles surrounding gene therapy in 2012, coinciding with the initial clinical trials of AAV-microdystrophin constructs for DMD[19]. These ethical considerations are arguably even more relevant today. Back in 2012, the potential for destructive immune-mediated myositis induced by locally or regionally administered compounds was projected as an important ethical dilemma. To justify safely moving forward with further human trials, the article referenced the ability of dogs to predict immunity in other diseases, such as hemophilia, a field to which Dr. Stedman has made significant contributions[20]. Reflecting his own support for using dogs to test efficacy and predict immunogenicity in gene therapy, Dr. Byrne has worked to develop a canine model of Pompe disease[21]. In their 2012 article, they concluded, "A convincing demonstration of durable efficacy following vascular delivery in a canine model would be reassuring".

Ultimately, the Kornegay laboratory[22] and others[23,24] have achieved sustained microdystrophin expression without toxicity in dystrophic dogs treated systemically with AAV vectors carrying a canine transgene. However, DMD patients treated with the same construct containing a human transgene at comparable doses experienced SAEs (as discussed below). A similar scenario occurred in XLMTM preclinical studies conducted by Childers and his colleagues, where myotubularin (*MTM1*)-deficient dogs treated with an AAV construct expressing the canine *MTM1* gene had remarkable improvement without toxicity[25], whereas some XLMTM patients developed fatal SAEs when the human transgene was used[26].

Dr. Michael Lawlor has assessed humans with DMD and XLMTM as well as genetically homologous animal models treated with AAV-transgenes[22,25-30]. He brings an essentially unique perspective on the phenotypic differences across these conditions, including the relative degrees of acute necrosis *vs.* chronic fibrosis and fatty deposition in DMD, as well as liver involvement in XLMTM. These microscopic changes, along with the variable underlying mutations, offer the most useful clues to explain SAEs seen in humans but not animal models[28,31].

SEVERE ADVERSE EVENTS (SAES) AND AAV-BASED CLINICAL TRIALS

Several recent reviews have described SAEs, including hepatotoxicity, thrombocytopenia, thrombotic microangiopathy (TMA), atypical hemolytic uremia syndrome (aHUS), myocarditis, and myositis,[2-7,26,31] linked to innate and adaptive immune responses as well as complement activation[32,33] in AAV-transgene clinical trials [Table 1]. The DMD trials used a miniaturized *microdystrophin* version of the *DMD* gene with portions of the rod domain removed to fit the limited AAV carrying capacity[3,5]. A full-length version of the *MTM1* gene was used in the XLMTM trial[26]. Similar adverse effects have been characterized in patients receiving AAV-transgene products for other neuromuscular diseases such as spinal muscular atrophy[5-9] and Pompe[5-9] and Danon[5] glycogen storage diseases. Less frequent systemic or ocular SAEs have been observed in hemophilia[7,34], lysosomal storage diseases (LSDs)[3], and degenerative retinal disorders[35], for which canine models have been used to demonstrate treatment efficacy and side effects[20,36-44].

Table 1. Adverse events in AAV-based neuromuscular disease clinical trials*

Disease	Company (product/transgene)	AAV serotype	Highest AAV dose (vg/kg)	Adverse events	*Additional references
DMD	Sarepta (elandistrogene moxeparvovec)	AAVrh74	1.33E14	Vomiting, liver injury with increased transaminases, rhabdomyolysis, myositis, myocarditis	Mendell *et al.*, 2023[176]
DMD	Solid Biosciences (SGT-101)	AAV9	2E14	Thrombocytopenia, renal damage, cardiopulmonary insufficiency, myocarditis	Dreghici *et al.*, 2024[177]
DMD	Pfizer (fordadistrogene movaparvovec)	AAV9	2E14	Thrombocytopenia, aHUS/thrombotic microangiopathy, myocarditis	Pfizer press release, 2024[178]
XLMTM	Astellas (resamirigene bilparvovec)	AAV8	3.5E14	Hepatic toxicity with hyperbilirubinemia, sepsis	Shieh *et al.*, 2023[26]
SMA	Novartis (onasemnogene abeparvovec)	AAV9	1.1E14	Fever, malaise, vomiting, acute liver failure, thrombocytopenia	Chand *et al.*, 2021[9]

*Data from Lek *et al.* (2022)[4] and Servais *et al.* (2023)[6]. DMD: Duchenne muscular dystrophy; XLMTM: X-linked myotubular myopathy; SMA: spinal muscular atrophy; aHUS: atypical hemolytic uremic syndrome; AAV: adeno-associated virus.

Cellular and humoral immunity to either the AAV vector or transgene product act together to induce these side effects[45] [Figure 1]. A range of risk factors could be involved, including the antigenicity of AAV capsid proteins and other construct components, the patient's genetic mutation, prior exposure to AAV and overall immune status, and product impurities resulting from the AAV manufacturing process[46]. Perhaps most importantly, the incidence of side effects has tended to correlate directly with AAV vector genome (vg) dose. Treatment effect has generally required dosing at 1-3E14[47], while toxicities in DMD and XLMTM have mostly occurred at or above a dosage of 1.1E14 vg/kg[4,33]. Obviously, there is a small therapeutic window that likely varies among patients.

Adaptive immunity against the transgene protein products also plays a role in DMD immunity. A collaborative study of patients with T cell-mediated reactions identified overlapping mutations in the dystrophin amino terminus region[4,31]. Exons 8 to 11 were deleted in all patient *DMD* genes, presumably causing the protein epitopes translated from the construct to be recognized as non-self-neoantigens [Figure 2] (see discussion of specific microdystrophin constructs below). This harkens back to immunity against dystrophin identified by Mendell *et al.* in their early AAV-microdystrophin DMD clinical trial[48].

Considering the role of complement in SAEs, markers should be monitored to track activation and response to treatment. In a study of TMA patients with SAEs after systemic AAV administration, immunoglobulin M (IgM) and IgG increased rapidly with an associated rise in D-dimer and a decline in the platelet count[33]. Activation of both classical and alternative complement pathways, as indicated by depleted C4 and elevated soluble C5b-9, Ba, and Bb antigens, occurred.

USING ANIMAL MODELS TO PREDICT TREATMENT SAFETY

The requirement that animals be used to demonstrate the safety (or toxicity) of new drugs dates to the Federal Food, Drug, and Cosmetic Act of 1938. Various regulations were ultimately developed by the US Food and Drug Administration (FDA), with guidelines by and large dictating that safety be demonstrated in two species, a rodent and non-rodent (often the dog), in both short- and long-term studies[49,50]. Recent reviews have suggested that only approximately 50% of animal studies, extending across species including dogs, predict drug toxicity[51]. Related to this observation and broader animal welfare concerns, the FDA Modernization Act 2.0 passed by the US Congress in 2022 removed the requirement for animal use in drug approval and stipulated that alternatives would be allowed[52,53]. This is in keeping with the 3Rs (replacement,

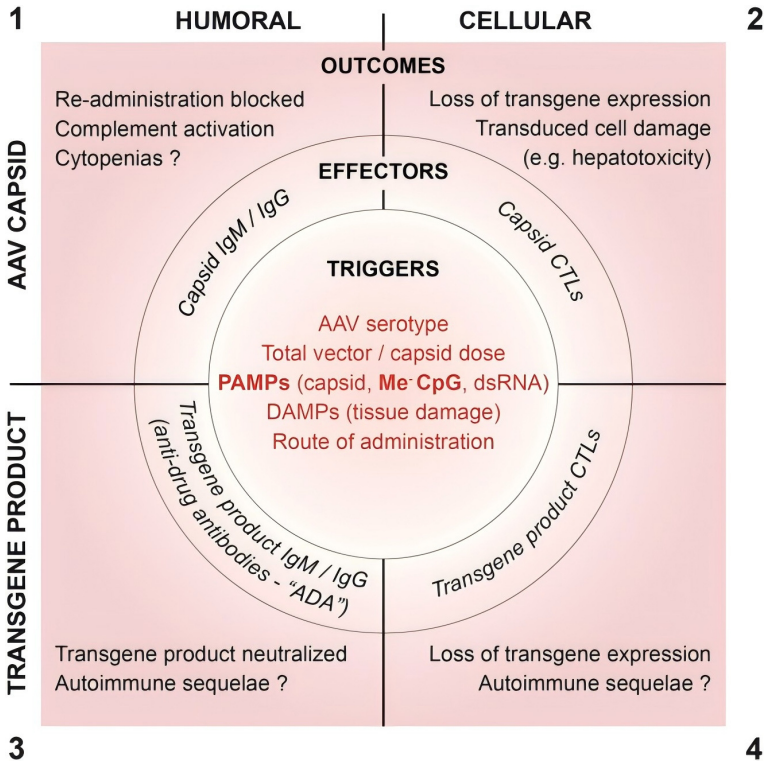

Figure 1. Schematic of four quadrants in which cellular and humoral immunity interact with AAV capsid and transgene proteins to cause SAEs. From Hamilton *et al.*, 2021[45]. Republished under Creative Commons Attribution License (CC-BY 3.0). AAV: Adeno-associated virus; SAEs: severe adverse events.

reduction, and refinement) approach toward the use of animals in research and could lead to greater reliance on alternatives like computational models, the use of recombinant antibodies produced in cell culture *vs.* live animals, and tissue engineering (*organ on chip*) technologies[54]. With that being said, pharmaceutical companies can still use animals to establish safety, and change is expected to occur gradually.

As shown by the SAEs in recent human trials, side effects associated with cell and gene therapy are generally more severe than those caused by traditional drugs. In their 2015 paper, Gopinath *et al.* emphasized the value of animal models in assessing variables of safety, efficacy, dosage, and localization of gene expression with viral vectors[55]. They stressed that choosing a suitable disease-specific model is of paramount importance for successful clinical translation. Consistent with this approach, the FDA has developed preclinical industry guidelines for cell and gene therapies, recommending 1) *in vitro* testing (e.g., functional assays, immunophenotyping, and morphologic evaluation) and *in vivo* pilot studies to establish the biological relevance of a specific animal species to the investigational product(s) before conducting definitive preclinical trials and 2) detailed assessment of the relevancy of each animal species used to support a potential clinical trial, with a summary of this assessment submitted as part of the preclinical section of the Investigational New Drug (IND) application[56-58]. Importantly, general adherence to these guidelines has not prevented recent SAEs in AAV clinical trials.

THE ROLE OF ANIMAL MODELS IN ASSESSING IMMUNITY TO AAV CONSTRUCTS

General considerations

The FDA guidelines mentioned above emphasize the importance of considering species-related

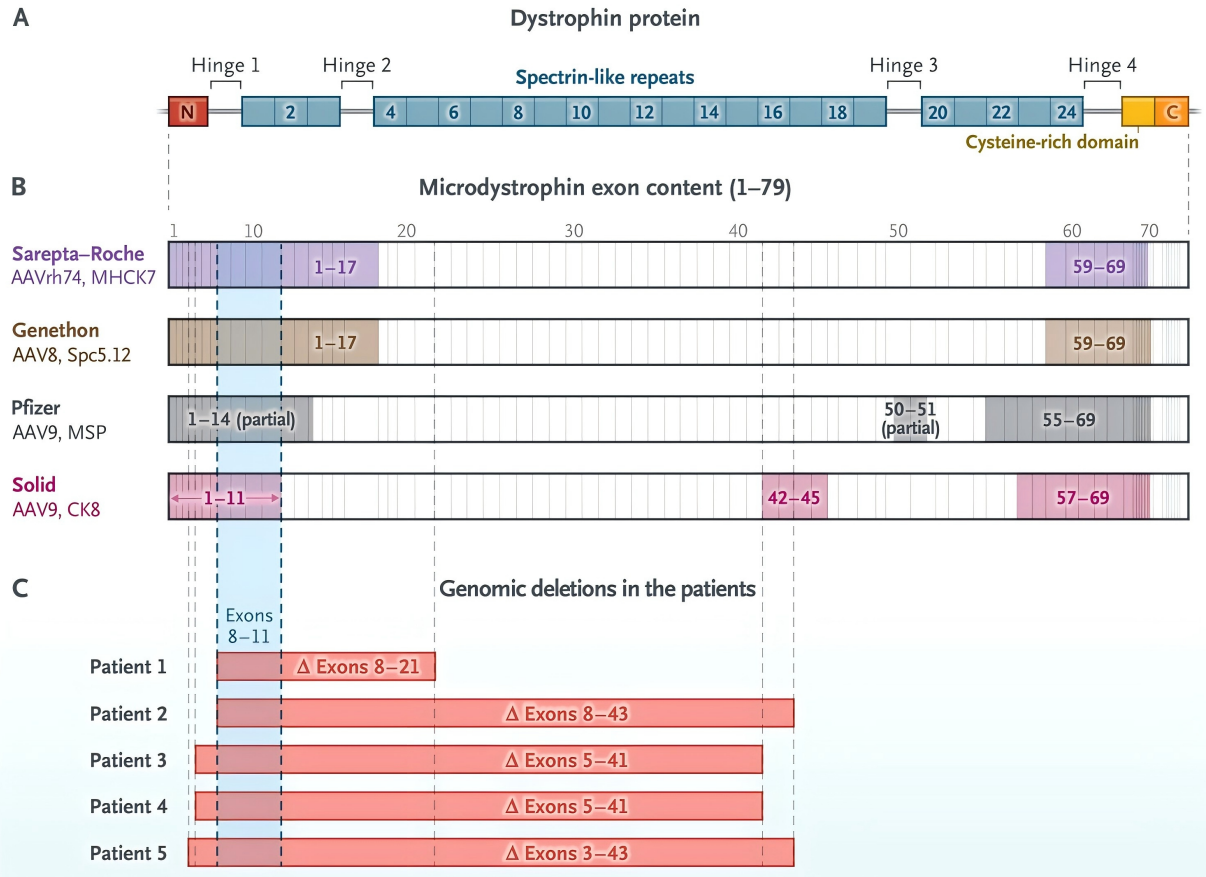

Figure 2. Diagram depicting DMD gene mutations associated with SAEs. Panels A, B, and C show the dystrophin structure, microdystrophin exon content of therapeutic transgenes, and patient mutations, respectively. From Bönnemann *et al.*, 2023[31]. Republished under the Rights Link for Scientific Communications through the Copyright Clearance Center. SAEs: Severe adverse events; DMD: Duchenne muscular dystrophy.

immunogenicity in gene therapy trials. Mice used for testing are often inbred with essentially identical genetic makeup. This contrasts with the genetic diversity among humans[59] and likely contributes to the limited ability of inbred mice to predict adverse effects of foreign and/or self-antigens[60]. On the other hand, large animals are reasonably outbred and mostly share physiological functions such as the immune system with humans[61]. In addition, they live comparatively longer, allowing for extended studies, and are similar in size to a neonate or small child so that scaling issues can be addressed. Important limitations include the relatively few well-defined genetic conditions in most large animal species, the high costs of housing and maintenance, difficulties in achieving adequate sample sizes due to longer gestation periods and smaller litter sizes, and concerns about animal welfare[62,63].

Dogs have been used to model a range of human diseases, from cancer[64] to genetic disorders[65]. Beyond the advantages offered by other large animal models, mutations have been identified in an increasing number of inherited diseases, allowing genetic therapies to be assessed[66]. Because pet dogs are treated as family members, sophisticated tests such as magnetic resonance imaging (MRI) and electrodiagnostic procedures routinely used in a veterinary specialty setting can be extrapolated to research models[67,68]. As an added bonus discussed further below, treatments developed in canine models are now benefitting companion dogs[69,70].

Regarding pre-existing immunity to AAV in dogs, one study noted particularly high neutralizing antibody titers to AAV6 in puppies, with levels then tapering[71]. Titers to AAV8 and 9 were less pronounced. Dystrophic dogs mounted a more robust antibody response to AAV, potentially because weaker suckling may have reduced immunoglobulin-induced immunity. Because AAV is a parvovirus, there has been interest in the effect of vaccination routinely employed against this virus in young dogs. Parvovirus vaccination status did not affect the AAV response. Another paper confirmed the selective increase in neutralizing antibodies to AAV6 and AAV1 in dogs[72]. Horses showed a particular increase in antibodies to AAV5, whereas pigs exhibited elevated levels of antibodies to several serotypes. Confirming the clinical significance of these antibodies, AAV transduction was much reduced in mice injected with serum from animals with high AAV titers.

In certain disease settings, dogs have predicted immunotoxicity better than mice and, in some cases, even more effectively than NHPs. The best example is hemophilia, for which naturally occurring models of factor VIII (hemophilia A) and IX (hemophilia B) have been studied for over 70 years[20,36-38]. Studies in canine hemophilia foresaw immunity to adenoviral vectors[37] and increases in transaminase enzymes subsequent to the administration of liver-directed AAV constructs[7]. Hepatocyte clonal expansion associated with AAV genomic DNA integration events after intravenous liver-directed therapy in hemophiliac dogs has raised concerns about long-term supraphysiologic factor levels and hypercoagulability[38]. Further supporting canine hemophilia as a model, companion hemophiliac dogs managed clinically with AAV-factor constructs have had occasional complications analogous to those in humans[69].

Several human clinical trials have utilized AAV-transgene constructs to correct inborn errors of metabolism that cause storage products to accumulate either systemically or predominantly in the central nervous system (CNS)[3]. As briefly mentioned earlier, viral vectors have also been used to treat humans with degenerative retinal diseases, wherein more limited ocular SAEs, often related to surgery instead of an immunologic reaction, have been reported[35]. Adverse events in LSD studies have not been well characterized, making it difficult to define the role animal models could play in their identification. Nevertheless, dogs with spontaneous LSDs[39-41] and degenerative retinal conditions[42-44] have been used to establish the therapeutic value of AAV-transgene constructs in advance of human trials. The construct has typically been given by intrathecal/intracerebral or intraocular administration, respectively, so inflammatory reactions would logically be localized. Adverse events related to the construct have not been reported in the LSD studies. Uveitis occurred after subretinal injection of an AAV-RPE65 construct in a canine Leber congenital amaurosis model[42].

Providing further support for the value of canine models to characterize post-treatment immunity, complement activation has been seen in hemophiliac dogs[73] and in canine models of hypersensitivity reactions[74]. A C5b-9 ELISA assay was used to test hemophiliac dogs. We saw hypersensitivity reactions in golden retriever muscular dystrophy (GRMD) dogs given Nemo Binding Domain (NBD) peptide to inhibit NF-κB but did not assess complement[75].

Preclinical studies for DMD and XLMTM

AAV-transgene constructs

Preclinical studies of AAV-microdystrophin constructs have followed a logical course from both a species and site of delivery perspective. As discussed in our 2012 review of the role of canine models in DMD[67], experiments were done initially in mdx mice[76,77], followed by dystrophic dogs[78-84]. Although there was a minimal immune response in mice, immunotoxicity was evident in dogs. Pioneering research at the University of Washington and Fred Hutchinson Cancer Center in Seattle, led by Jeff Chamberlain, Stephen

Tapscott, and Rainer Storb, determined that intramuscular injection of AAV2 and AAV6-microdystrophin constructs elicited a marked cytotoxic T cell response to capsid proteins[78,79]. Studies by the Takeda laboratory in Japan involving beagle dogs carrying the GRMD mutation, injected with an AAV2-CMVLacZ construct, showed an immune response against the β-galactosidase protein, suggesting that dystrophin could act as a neoantigen[80]. Indeed, non-immunosuppressed GRMD dogs treated with an AAV construct containing a codon-optimized *human* microdystrophin by the Kornegay laboratory developed marked myositis [Figure 3][81] that was not seen in subsequent studies using the same canine gene[82]. This suggested divergent amino acid sequences between species could lead to immunity. Other studies revealed that the immune response to AAV-microdystrophin constructs in dystrophic dogs was lessened by immunosuppression[80,83] and largely absent even without immunosuppression when a canine AAV-microdystrophin and muscle-specific promoter were used[84]. Results from contemporaneous phase 1 human trials of AAV-microdystrophin constructs injected intramuscularly were mixed, with one having no evidence of immunity[85], whereas another had a dystrophin-specific T cell response[48].

Based on generally encouraging results of intramuscular delivery of AAV-microdystrophin constructs in dogs, work was extended to high-pressure transvenular regional limb delivery using the Bier block approach applied clinically for local anesthesia and pain relief in the distal limbs[86,87]. Hansell Stedman and his colleagues at the University of Pennsylvania had previously utilized this technique to deliver AAV-factor IX transgene constructs to hemophiliac dogs based on the premise that the factor produced in muscle would move systemically to correct the clotting defect[88]. Striking dystrophin expression was found in dogs using this regional limb delivery technique without immunosuppression, showing more broadly the feasibility of vascular delivery[24,67,89]. The relative absence of an immune response suggested venous injection might actually be less immunogenic, presumably because muscle injury was avoided and antigen was less concentrated. This regional limb technique was then used to safely deliver saline to adult muscular dystrophy patients by colleagues at the University of North Carolina-Chapel Hill[90]. The next logical step would have been to apply the approach in DMD. However, with encouraging results already being established for generalized intravenous delivery of AAV-microdystrophin constructs in dystrophic mice[91,92] and dogs[22-24], research moved towards systemic administration. Canine studies have provided guidance for dosing human AAV constructs. For instance, our GRMD AAV9-microdystrophin study included 1E13, 1E14, and 2E14 vg/kg dosage groups[22], while DMD patients in the Solid Biosciences SGT-001 trial were treated at 5E13 and 2E14 vg/kg.

There is not a naturally occurring dystrophin-deficient NHP model, but CRISPR/Cas9 has been used to disrupt the *DMD* gene in rhesus monkeys[93] and other species including pigs, rabbits, and rats[94]. Mechanistic studies at the cellular level were done in monkeys, potentially providing a platform for treatment development[95]. Importantly, given that mosaic mutations are generated with CRISPR, care must be taken in interpreting findings[94]. A systematic histopathologic natural history study of dystrophic monkeys would be necessary to determine whether this model is phenotypically analogous to DMD, and the associated likelihood preclinical studies will translate to patients.

Work has also been done in normal NHPs to establish proof-of-concept. As an example, encouraging results were obtained using regional limb delivery of AAV-microdystrophin constructs in cynomolgus macaques[96]. In addition, plasmapheresis lowered anti-AAV antibodies in NHPs with pre-existing AAV immunity[97]. Moreover, there has been natural interest in using monkeys to predict immunity. Dr. Wilson's lab at the University of Pennsylvania identified sensory neuron/dorsal root ganglia toxicity in pigs and hepatotoxicity in NHPs receiving a dose of 2E14 AAV9-human survival of motor neuron (SMN) protein[98]. A more recent NHP study from his lab detected hepatic microvasculature toxicity after systemic

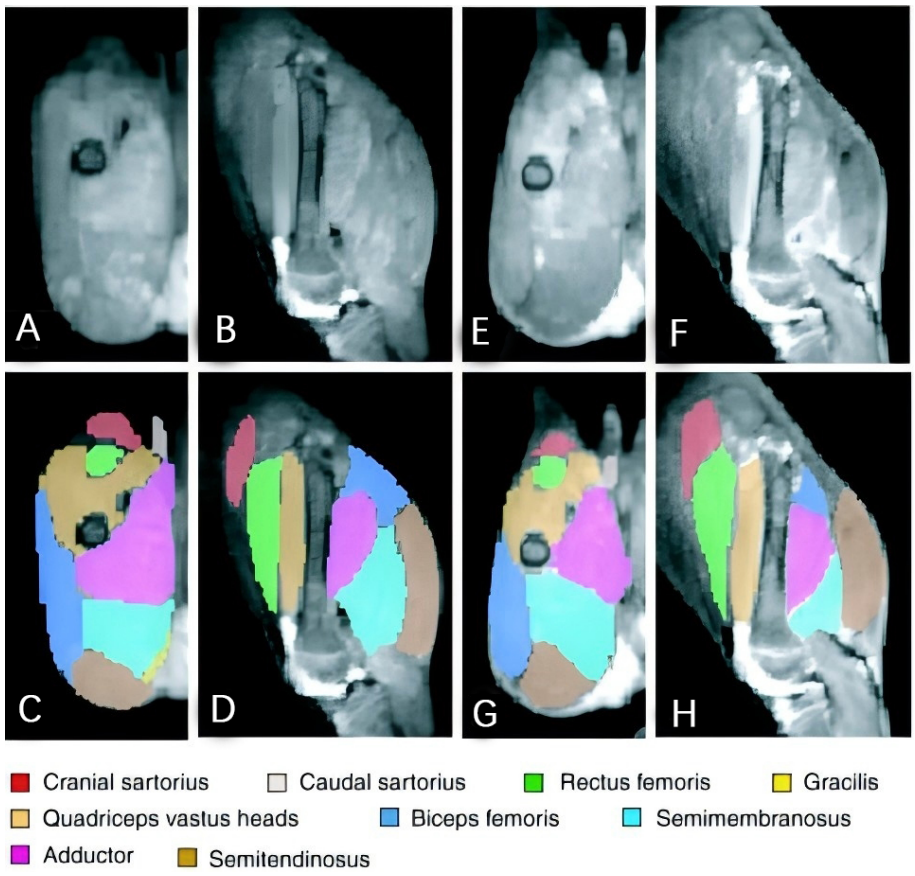

Figure 3. T2-weighted magnetic resonance images of pelvic limb muscles 8 weeks after AAV9-CMV-mini-dystrophin vector intravenous injection of two GRMD dogs (A-D, E-H) at 4 days of age. Images were segmented and color-coded to outline individual muscles. Signal-intense lesions are particularly pronounced in the vastus heads of the quadriceps and adductor muscles. These changes persisted with fat saturation, suggesting that they most likely represent fluid due to inflammation or edema. From Kornegay *et al.*, 2010[81]. Republished under Creative Commons Attribution License (CC-BY 3.0).

administration of AAV-enhanced green fluorescent protein constructs at doses ≥ 1E14 vg/kg[99].

Regarding preclinical XLMTM studies, *MTM1* knockout mice had robust clinicopathologic improvement over a 6-month period after a single tail vein dose of 1E13 AAV8-*MTM1*[100]. Comparable improvement was seen in a subsequent canine XLMTM study, in which AAV8c*MTM1* was dosed in three groups (n = 3/each; 0.3E14, 2E14, and 5E14 vg/kg). Results were compared to those from six untreated controls. Dogs were followed for 17 weeks post-infusion, at which time all untreated controls had died.

Cell-based therapies

As emphasized recently by Graves and Storb[101], "It is difficult to imagine the successful development of hematopoietic cell transplantation (HCT) without the significant contributions made by the canine preclinical animal model." Studies in normal outbred dogs featured prominently in several advancements, including the importance of histocompatibility matching, establishing long-term donor hematopoietic cell engraftment, prevention of graft-*versus*-host disease, advancing effective conditioning and post-grafting immunosuppression protocols, and applying HCT to induce tolerance for solid organ and composite tissue transplantation[101,102]. Encouragingly, similar HCT protocols are now being used to manage companion dogs with hematological disorders and lymphoproliferative malignancies[101-103].

Based on promising results in the late '90s of HCT in mdx mice[104] and a single DMD patient treated for concomitant severe combined immune deficiency[105], there was keen interest in using HCT in DMD. Given that much of the pioneering canine work on HCT was done at the Fred Hutchinson Cancer Research Center, GRMD/CXMD carriers from the University of Missouri and Cornell were used to establish a colony in Seattle. However, in a subsequent study, dystrophic dogs with allogenic bone marrow engraftment did not have increased dystrophin-positive fibers, wild-type dystrophin RNA, or donor-derived myonuclei[106].

This scenario whereby canine studies did not ultimately confirm findings from mdx mice had precedent from myoblast transplantation studies done in the 1980s and '90s. Based on proof-of-principle studies in mdx mice[107], human trials were instituted with disappointing results[108,109]. We had characterized canine myoblasts[110] and conducted analogous transplantation studies in GRMD dogs with little success, turning instead to an autologous cell model preceded by muscle injury[67,111]. Failed translation from mice to humans (and dogs) probably related to variables influenced by scale, such as cell migration[112] and the combined effects of poor blood supply and immune rejection[113,114].

Why were SAEs absent in canine DMD and XLMTM models?

Considering dogs have predicted the outcome of gene and cellular therapy in other settings, why did genomically homologous canine models not identify SAEs seen in DMD and XLMTM clinical trials using comparable AAV doses? Immunity to either the transgene product (dystrophin or myotubularin) or AAV vector could be involved.

The GRMD model has a single base change in the 3' consensus splice site of intron 6, causing exon 7 to be skipped and termination of the dystrophin reading frame in exon 8[115]. Alternative splicing can restore the reading frame, leading to revertant fibers that could protect against anti-dystrophin immunity[116]. Thus, GRMD dogs may not be prone to the immune reaction to dystrophin evident in DMD boys with mutations in the N-terminal region[31]. Interestingly, the null mutation in the dystrophic German shorthaired pointer model does not allow for alternative spicing and might better predict an immune response against dystrophin (see discussion of the immune response to dystrophin and utrophin in the pointer model below)[117,118].

An analogous situation could exist in XLMTM. Truncating mutations in the human *MTM1* gene are associated with a more severe form of the disease compared to non-truncating inframe deletions/insertions and missense mutations[119-121]. Affected dogs have a missense mutation[27], allowing protein production that could establish tolerance. Moreover, as with utrophin in DMD (see below), AAV constructs carrying MTM-related protein (MTMR) transgenes have therapeutic benefits in XLMTM[122,123]. However, mutation type did not appear to contribute to the incidence of SAEs in the XLMTM ASPIRO trial, with no disproportionate increase in myotubularin antibodies seen in patients with null mutations[26].

To ensure that findings from a genomically *homologous* animal model are optimally translatable to humans, the model must be phenotypically *analogous*[124]. While both GRMD and canine XLMTM express disease phenotypes similar to those of their human disease counterparts, they are not completely analogous[125] [Figure 4]. One key difference relates to the chronology of disease embodied within the relative longevity of dogs and humans. Based on an epidemiological study of dogs admitted to veterinary medical teaching hospitals, year one of a golden retriever's life is roughly equivalent to the first 20 years for a human[126]. For the sake of clinical extrapolation, we have divided these periods into four age groups, with 3, 6, 9, and 12 months in GRMD corresponding to 5, 10, 15, and 20 years in DMD[127]. The GRMD and DMD disease phenotypes generally parallel one another for the first 6 months and 10 years, respectively, with each having

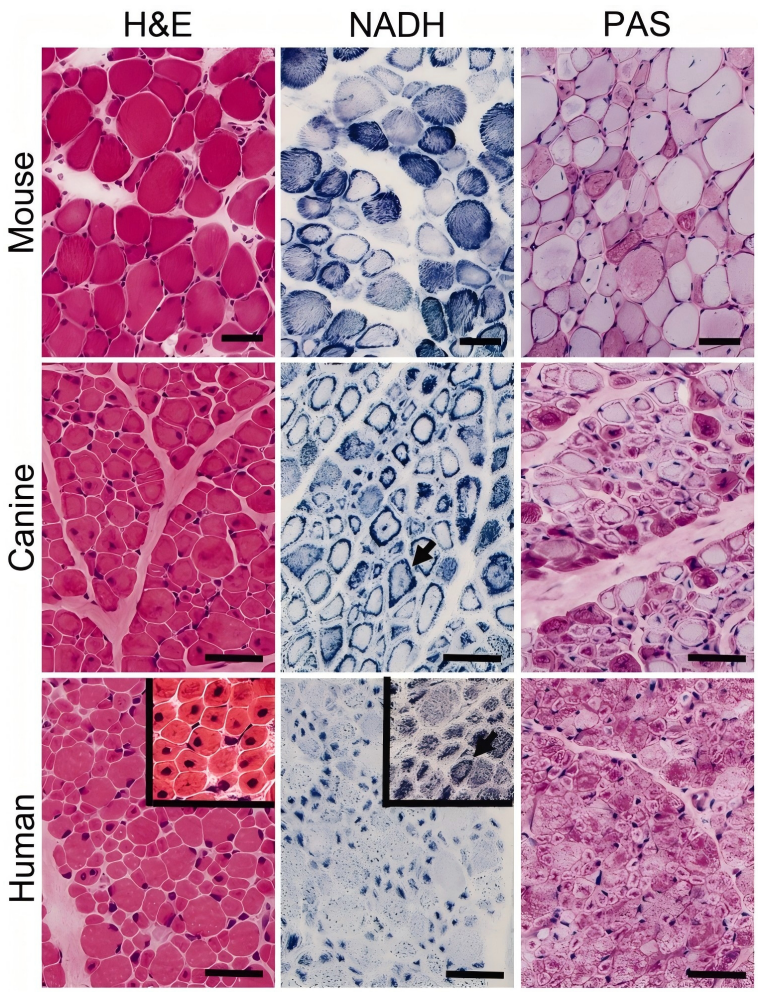

Figure 4. Comparative XLMTM histopathologic lesions in a *MTM1* knockout mouse (43 days, mid-stage), dog (18 weeks, end-stage), and boy (2 months, terminal-stage). Increased numbers of central nuclei (inset in human sample) and fiber size variation due to numerous hypotrophic and atrophic (human) fibers are seen on H&E and PAS stains. Organelle (NADH) and glycogen (PAS) mislocalization is evident as either peripheral halos (human) or dark peripheral staining (dog and mouse). "Necklace fibers" (arrows) are seen in canine, some human patient biopsies (NADH inset), and rare mouse fibers. Scale bar = 40 µm. From Lawlor *et al.*, 2016[28]. Republished under Creative Commons Attribution License (CC-BY 3.0). XLMTM: X-linked myotubular myopathy.

marked clinicopathologic progression. After 6 months, clinical signs and histopathologic lesions [Figure 5] in GRMD tend to stabilize. Affected dogs often live well into adulthood, whereas DMD patients continue to deteriorate[67,125,127]. Progressive histopathologic disease and associated inflammation in DMD patients[128,129] receiving AAV constructs could predispose them to complement activation and associated SAEs[6]. One particular distinguishing histopathologic feature between DMD and GRMD is the degree of fatty deposition, with affected dogs having markedly less intramuscular fat than humans based on both histopathologic[130,131] [Figures 5 and 6] and MRI assessment[132,133]. Given the role that cytokines secreted by adipocytes (adipokines) play in inflammation[134,135], greater fatty infiltration in DMD boys might predispose them to a more marked immune response.

The clinical course of XLMTM in Labrador retrievers is more rapid than in GRMD, consistently causing progressive weakness and muscle atrophy, necessitating euthanasia by 4-6 months of age[125,136]. Nonetheless, considering XLMTM in dogs is caused by a missense mutation[27], the phenotype is likely comparatively

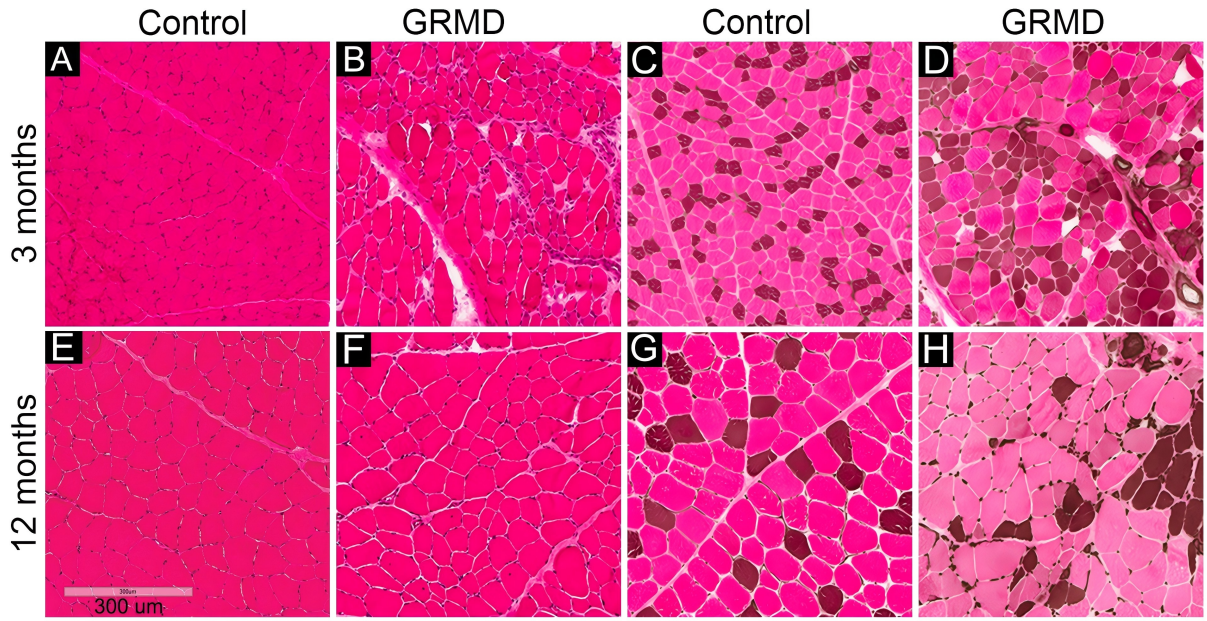

Figure 5. Photomicrographs of H&E (A, B, E, and F) and acidic ATPase (pH 4.3) (C, D, G, and H) stains of the vastus lateralis muscle of GRMD *vs.* normal dogs at 3 and 12 months, roughly equivalent to 5 and 20 human years based on comparative longevity studies[126], showing relative stabilization of histopathologic lesions, with minimal fatty deposition in GRMD. Note also the increase in type I (darker stained) fibers with some grouping in GRMD. Although there is minimal open space suggestive of fat in GRMD at 12 months, the endomysial connective tissue separating individual myofibers is increased (see Fan *et al.*, 2014[132] for GRMD images at 6 months and Figure 6 for comparison to DMD). Courtesy of Drs. Jane Fan and Yael Shiloh-Malawsky, UNC-Chapel Hill.

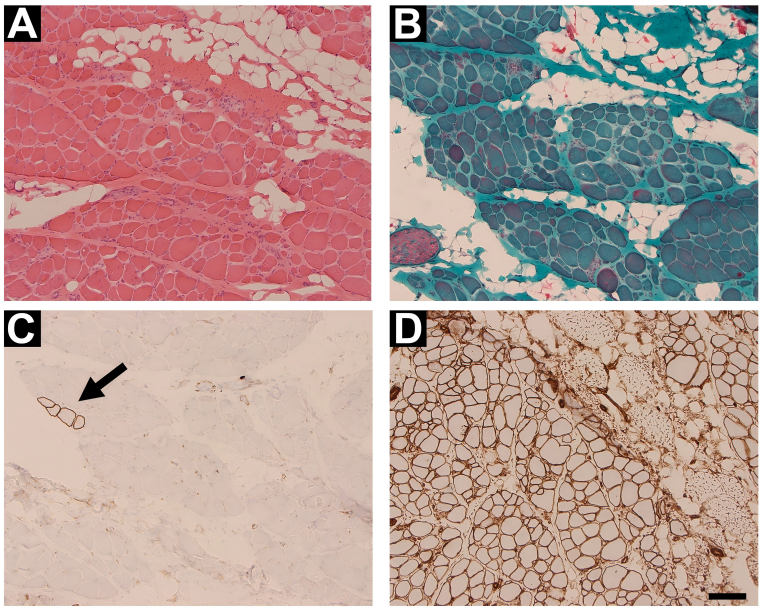

Figure 6. Photomicrographs of (A) H&E, (B) GT staining for collagen and connective tissue, (C) Dys2 dystrophin, and (D) utrophin histochemical stains of the vastus lateralis muscle from a 13-year-old boy roughly equivalent to an 8-month-old dog based on comparative longevity studies[126]. In the H&E (A) and GT (B) stains, the endomysial connective tissue separating individual myofibers and open spaces typical of adipose tissue (fat; top right) are increased (see Figure 5 for GRMD comparison). Note also the small cluster of dystrophin-positive ("revertant") fibers in C (arrow) and prominent utrophin staining consistent with upregulation. Otherwise, there is variation of myofiber size with occasional internal nuclei, both nonspecific features of myopathy, with little to no necrosis or inflammation. Scale bar = 50 μm. GT: Gomori trichrome.

milder than in affected humans with truncating mutations. This is substantiated by the 4- to 6-month course of XLMTM in Labradors compared to the rapid deterioration of human infants over a period of only days to weeks[120,137]. More importantly, dogs do not develop cholestatic hepatobiliary disease that seemingly predisposes human XLMTM patients to SAEs[26,138-140].

In light of their historical use to demonstrate efficacy and immunogenicity with gene and cell therapy and as evidenced by recent papers[141,142], canine models will probably continue to be used in AAV-transgene preclinical testing. To better predict future problems, thought should be given to tracking early subclinical markers of the innate immune response, especially complement activation. As precedent, complement C8 alpha chain and several other serum proteins were elevated in subclinical leishmaniasis in dogs and tracked with disease progression[143].

Background on AAV-microdystrophin constructs in DMD clinical trials [Figure 2]

The Chamberlain laboratory has systematically studied structural modifications of the dystrophin central rod domain to identify microdystrophins with maximal functional efficacy[144]. They chose a particular design, named μDys5, for further study because of its functional performance and small size. These studies have been augmented by work from Stephen Hauschka on regulatory cassettes showing that the CK8e cassette consistently drives more muscle-restricted protein expression[145]. Ultimately, μDys5 was coupled with CK8e for clinical development as SGT-001 in collaboration with Solid Biosciences (NCT03368742).

The improved function seen with the CK8e-μDys5 construct can be attributed to several factors[144] (Jeff Chamberlain, personal communication, 2022). First, μDys5 has five spectrin-like repeats (SRs) and two hinges as opposed to combinations of four or six SRs and three hinges in the other seven transgenes tested. Reasons to account for greater force generation using a transgene with fewer (five *vs.* six) SRs are not clear and were not specifically pursued in these studies. One explanation could relate to the replacement of hinge 3 in μDys5 with the entire SR23 to enhance stability and elasticity. Perhaps most importantly, μDys5 contains the nNOS binding domain found by Duan *et al.* to be within dystrophin SRs 16 and 17[146]. Mouse models lacking or having a mislocalized nNOS domain are at risk for functional ischemia with associated fatigue[147]. Moreover, Becker muscular dystrophy (BMD) patients with exon 42-45 deletions that include repeats 16 and 17 show mislocalization of nNOS in muscle biopsy samples and a more severe phenotype[148].

Comparing the microdystrophins that have been used in the five DMD clinical trials discussed here, only rAAV9-CK8e-μDys5 (SGT-001) contains the R16/17 SRs. Notably, the study of eight microdystrophins mentioned above[144] included a close variant (μDysH3) of an earlier transgene (μDysH2) currently used in the Sarepta and Genethon clinical trials[149]. As opposed to the five SRs and two hinges in μDys5, μDysH2 has four SRs and three hinges[149,150]. Only two of the five SRs in μDys5 are included in μDysH2. Finally, while μDys5 is driven by the CK8e cassette, the Sarepta construct employs MHCK7 and Genethon uses the synthetic Spc5-12 muscle-specific promoter[150]. Continuing the comparison, the microdystrophin in the Pfizer clinical trial was developed and tested by Xiao Xiao in both dystrophic mice[76] and dogs[81]. Called △3990, this transgene has five SRs and three hinges. A canine variation (△3849) lacking the central hinge was efficacious in mdx mice[151]. The Pfizer construct includes a muscle-specific creatine kinase promoter[152].

Ongoing DMD clinical trials are further distinguished by the AAV serotype used. The Solid and Pfizer constructs employ AAV9, whereas Genethon uses AAV-8. Sarepta utilizes AAV-rh74, which is like AAV8[149,150].

A recent clinical trial by REGENXBIO utilizes a microdystrophin that includes four SRs and three hinges plus an extended coding region of the carboxyl-terminal (CT) domain[153]. The binding of α-dystrobrevin and α-syntrophin by this CT domain may help recruit nNOS. The RGX-202 construct uses a novel AAV8 vector (NAV) Technology Platform and a muscle-specific promoter (Spc5-12)[153]. Strength measurements, histopathologic findings, immunostaining for nNOS, and MRI studies from mdx mice treated with RGX-202 suggested improvement[153].

THE WAY FORWARD

General considerations

Several expert panels offering perspectives from scientists, clinicians, regulatory bodies, funding agencies, patient advocacy groups, and industry have been convened to address the causes and mitigation of SAEs after systemic AAV-construct administration[4,5,8,33]. Given the critical role played by AAV dose in these reactions, effort has been given to engineer myotropic serotypes effective at lower doses with fewer off-target side effects compared to naturally occurring AAVs[154]. One high-throughput approach combined DNA/RNA barcoding with multiplexed next-generation sequencing to identify an AAV9 mutant called AAVMYO that specifically targets the musculature[155]. In another study, bioengineered AAV serotypes similar to AAV9 effectively targeted muscle in *MTM1* knockout and mdx mice at lower doses than those necessary for standard AAV9 constructs[156]. Myotropic AAVs continue to be defined[157,158].

Importantly, the seroprevalence of antibodies against these bioengineered capsids was comparable to AAV9, suggesting they would translate to the clinic. Work done simultaneously by Byrne and his collaborators at the University of Florida showed that capsid- and genome-modified AAVrh74 vectors, originally isolated from rhesus monkeys, were more efficient than AAVrh74 controls in transducing a reporter gene in the muscle of wild-type mice[159] [Figure 7]. Providing further encouragement, titers to nonoptimized rAAVrh74 are typically negative or low in DMD patients, suggesting they may be less immunogenic[160], and this vector has been used successfully in DMD microdystrophin clinical trials[161]. To allow for a more streamlined pathway to the clinic, AAV vectors with cross-species applications have been developed[162].

Since the *DMD* transgene and translated dystrophin protein contribute to the immune response, mutation exclusion criteria are being critically assessed[31]. This continues a trend started for the Serepta clinical trials in which patients with *DMD* gene deletions in exons 8 or 9 are excluded and those with deletions in any of the exons included in the microdystrophin transgene are cautioned about an increased risk of severe immune-mediated myositis. Additional strategies have been directed at improving the efficiency of gene expression or reducing exposure to immunogenic dystrophin. For instance, studies have indicated that self-complementary single-stranded AAV vectors bypass the requirement for viral second-stranded DNA synthesis, thus lowering the required AAV dose[159,163]. Additionally, in studies completed jointly in the Stedman and Kornegay labs, the use of utrophin in mdx mice and the dystrophin null canine pointer model eliminated the T cell immune response[118] [Figure 8]. Subsequent reviews have shown potential structural and immunological advantages of utrophin, while emphasizing the need to consider amino acid sequences and domains as has been done with microdystrophins[164].

Given the potential for mutation type to have an analogous impact on immune response in XLMTM patients receiving AAV-*MTM1* constructs, consideration may also be given to using *MTMR2* or other *MTMR* transgenes[26,122,123]. With that being said, critical surveillance for and exclusion of patients with pre-existing cholestatic liver disease will be most important.

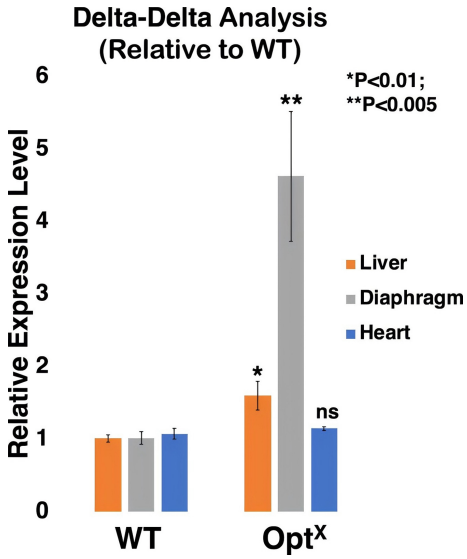

Figure 7. Histogram of comparative GFP transgene RNA expression of WT *vs.* capsid and genomic optimized (Opt^X) AAVrh74 vectors injected IV into C57BL mice. Bar graphs show average of WT- and Opt^X-AAVrh74 △Ct across organs. Statistical significance was determined between WT and Opt^X (see error bars and *P* values). From Shoti *et al.*, 2023[159]. Republished under Creative Commons Attribution License (CC-BY 3.0). WT: Wild-type.

Whether immunity is generated against AAV capsid or transgene proteins, it is essential to carefully consider immunomodulatory strategies to further reduce the risk of side effects. A variety of compounds have been used, ranging from prednisolone alone to more targeted combinatory approaches with drugs like the CD20 inhibitor rituximab[33] and complement blocker eculizumab[46].

Regarding the limitations of animal studies in predicting immune reactions, the expert panels and others have emphasized the importance of choosing species whose disease phenotype and immune functions closely resemble those of humans[46]. These findings remind us of the long-held principle that there is no perfect animal model. Indeed, in their 2020 review, Martino and Markusic concluded, "… humans will probably be the best animal model to study AAV immunity with experience gained from the use of approved AAV biologics and clinical trials[165]."

AAV-microdystrophin studies

In the ongoing microdystrophin clinical trials, SAEs have largely been manageable, although the functional effects have been mixed. Sarepta's Elevidys (SRP-9001; delandistrogene moxeparvovec) received FDA approval in the United States in June 2023. The Embark clinical trial did not reach the primary endpoint of the North Star Ambulatory Assessment (NSAA) test, but significant benefits were observed in secondary outcomes compared to placebo. Notably, regarding the unmet needs of non-ambulatory DMD boys and young men discussed above under ethical considerations, the FDA somewhat controversially recently granted accelerated approval for the use of Elevidys in this patient group[166].

Working in collaboration with Sarepta to treat patients in Europe, Genethon has reported promising results with their microdystrophin construct, GNT0004, which employs the same transgene as Sarepta's Elevidis, with an AAV8 serotype and a different promoter.

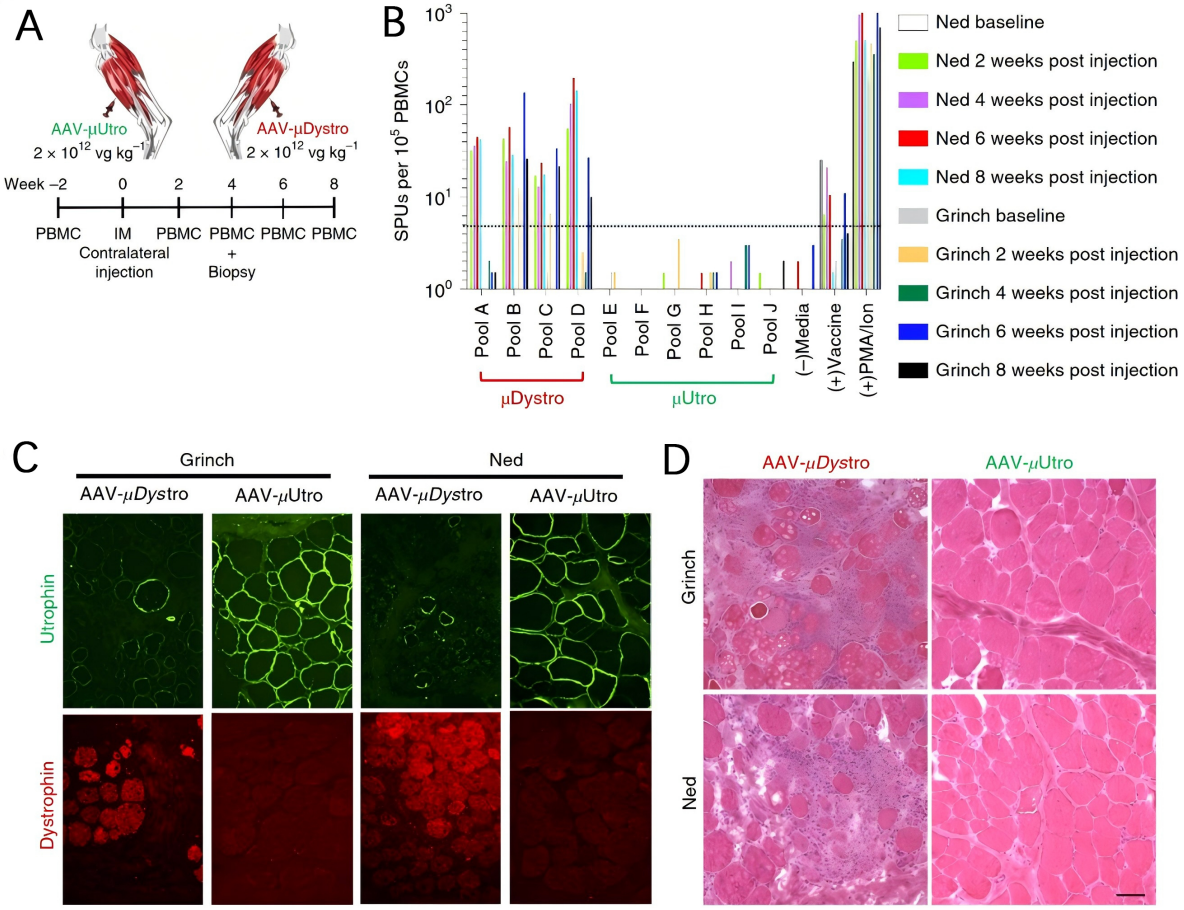

Figure 8. Schematic (A) of two German shorthaired pointer muscular dystrophy dogs (Ned and Grinch), in which the opposite cranial tibialis muscles were injected with either µ-dystrophin or µ-utrophin, showing the numbers of blood mononuclear cells reacting over time when cultured with peptides spanning each protein (B), together with the level of µ-dystrophin or µ-utrophin gene expression (C) and resulting histopathologic lesions (D). Note the marked inflammatory cell response in the AAV-µ-dystrophin treated limb (left) contrasted with the absence of inflammation in the AAV-µ-utrophin limb (right). From Song *et al.*, 2019 (Stedman HH, corresponding author)[118].

Solid Biosciences has moved forward with a clinical trial (NCT06138639) of their new construct, SGT-003, which incorporates a proprietary myotropic capsid, AAV-SLB10, while continuing to include the nNOS-binding R16 and R17 SRs from their original AAV9-based SGT-001. Based on data from mdx mice and NHPs, SGT-003 provides greater microdystrophin delivery to skeletal muscles and the heart.

In contrast, Pfizer recently released negative clinical data from its Phase 3 CIFFREO clinical trial (NCT04281485) of fordadistrogene movaparvovec. Treated DMD boys did not reach the primary endpoint on the NSAA test nor differ significantly compared to placebo regarding secondary outcomes. They have paused dosing due to a fatal serious adverse event in the phase 2 DAYLIGHT trial (NCT05429372).

In the RGX-202 clinical trial (NCT05693142), a three-patient cohort has been treated at a dose of 1E14 vg/kg, with a second group planned at 2E14[167,168]. Limited preliminary functional data are encouraging, and no SAEs have been reported.

Overall, these results are promising, but they highlight that the microdystrophin treatment strategy, by definition, is suboptimal. A miniaturized partially functional version of dystrophin is used and the duration and distribution of its expression remain to be well defined in patients[169-171]. A further concern was recently raised by studies showing accelerated heart involvement in a murine model with a severe cardiac phenotype, apparently due to competition between microdystrophin and utrophin at the cardiomyocyte membrane[172]. Accordingly, while efforts to optimize the microdystrophin treatment approach continue, studies of other potentially curative genetic and stem cell therapies will be pursued[169,173,174], coupled with drugs to lessen secondary effects of dystrophin deficiency such as inflammation and fibrosis[175].

AAV-*MTM1* studies

The SAEs associated with AAV-*MTM1* were seen in the ASPIRO clinical trial (NCT03199469) of resamirigene bilparvovec (AT132; rAAV8-Des-hMTM1) conducted initially by Audentes Therapeutics[26]. Astellas Pharma Inc. acquired Audentes in 2020 and subsequently placed a voluntary hold on dosing additional patients due to the SAEs. As part of an effort to develop alternatives for XLMTM treatment, Astellas recently licensed a new gene therapy, KT430, developed by Kate Therapeutics. KT430 utilizes a muscle-targeting myoAAV vector to deliver the *MTMI* gene[157].

CONCLUSION

Limitations of AAV-based therapies remind us that there is no perfect preclinical model. Gene therapy will always carry certain risks and is unique in that the effects of the drug are irreversible once delivered. The treating clinician must balance the basic medical tenet of "first, do no harm" against the inevitable progression of diseases like DMD and XLMTM. Here, careful attention must be paid to the perspective of the patients and their parents.

DECLARATIONS

Authors' contributions

Conceived the general approach and wrote the first draft of the paper: Kornegay JN
Reviewed the paper: Stedman HH, Lawlor MW, Byrne BJ, Childers MK

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Kornegay JN (Texas A&M) and Byrne BJ (University of Florida) laboratories received research support from Solid Biosciences Inc. for studies by Birch *et al.* (2023). Kornegay JN was a paid consultant for Solid Biosciences Inc. Stedman HH is a co-founder of StrongHolt Therapeutics and an inventor on related patents in the field of gene therapy for muscle disease. Childers MK is an inventor of a patent on gene therapy for myotubular myopathy. Lawlor MW is the founder, CEO, and owner of Diverge Translational Science Laboratory. Lawlor MW is or has recently been a member of advisory boards for Solid Biosciences, Taysha Gene Therapies, and Astellas Gene Therapies (formerly Audentes Therapeutics). Lawlor MW is also a consultant for Astellas Gene Therapies (formerly Audentes Therapeutics), Encoded Therapeutics, Modis Therapeutics, Lacerta Therapeutics, Dynacure, AGADA Biosciences, Affinia Therapeutics, BioMarin, Locanabio, Vertex Pharmaceuticals, Voyager Therapeutics, and Entrada Therapeutics. Lawlor MW receives or has recently received research support from Astellas Gene Therapies, Solid Biosciences, Kate Therapeutics, Prothelia, Ecogenome, Cure Rare Disease, Rocket Pharma, Ultragenyx, Carbon Biosciences,

Locanabio, Regenxbio, Vita Therapeutics, Lexeo Therapeutics, Lovelace Biomedical, Prevail Therapeutics, and Satellos.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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