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Review

Peptide-conjugate antisense based splice-correction for Duchenne muscular dystrophy and other neuromuscular diseases

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ABSTRACT

Duchenne muscular dystrophy (DMD) is an X-linked disorder characterized by progressive muscle degeneration, caused by the absence of dystrophin. Exon skipping by antisense oligonucleotides (ASOs) has recently gained recognition as therapeutic approach in DMD. Conjugation of a peptide to the phosphorodiamidate morpholino backbone (PMO) of ASOs generated the peptide-conjugated PMOs (PPMOs) that exhibit a dramatically improved pharmacokinetic profile. When tested in animal models, PPMOs demonstrate effective exon skipping in target muscles and prolonged duration of dystrophin restoration after a treatment regime. Herein we summarize the main pathophysiological features of DMD and the emergence of PPMOs as promising exon skipping agents aiming to rescue defective gene expression in DMD and other neuromuscular diseases. The listed PPMO laboratory findings correspond to latest trends in the field and highlight the obstacles that must be overcome prior to translating the animal-based research into clinical trials tailored to the needs of patients suffering from neuromuscular diseases.

Contents

Abbreviations: ASO, antisense oligonucleotides; CNS, central nervous system; CPP, cell penetrating peptide; DGC, dystrophin glyco-protein complex; DMD, Duchenne muscular dystrophy; FDA, US food and drug administration; PMO, phosphorodiamidate morpholino; PPMO, peptide-conjugated PMOs; PS, phosphorothioate; SMA, Spinal muscular atrophy; 2′-OMe, 2′-O-methyl; 2′-MOE, 2′-O-methoxyethyl; 6MWT, 6-minute walk test.

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1. Introduction - Duchenne muscular dystrophy

1.1. Clinical presentation

Duchenne muscular dystrophy (DMD) is an X chromosome-linked, progressive, fatal degenerative muscle disorder affecting approximately 1/3,500-5,000 male births worldwide [1–3]. Signs of disease are visible early in childhood (2-5 years) and comprise a delayed ability to walk or waddling gait, impairment in movements, difficulty in running and frequent falls [4,5]. Progressive muscle weakness and joint contractures lead to loss of ambulation and wheelchair dependency around the age of 9-12 [6]. Scoliosis is a frequent complication that starts developing as soon as the loss of autonomous ambulation occurs and causes a significant negative impact on the respiratory system [7,8]. Restriction of diaphragmatic movements and pulmonary expansion further compromises respiratory function and around the age of 20, mechanical ventilation may be necessary to sustain life [9,10]. Myocardial impairment originates in the inferolateral wall and progressively leads to left ventricular fibrosis and dysfunction [11,12]. A cognitive impairment component of DMD with deficits in short term memory, multitasking, procedural learning and problem-solving is attributed to dysfunctions in cerebro-cerebellar pathway and has recently become the topic of thorough investigation [13–15]. Affected individuals succumb due to repeated pulmonary infections arising from mechanical support and/or cardiac muscle impairment [16].

While most DMD patients did not reach adolescence in the 1970s, improvement in current pharmaceutical regimes means that many of them may live up to their fourth decade [17]. However an extension of life expectancy is achieved through adequate and timely management of cardiopulmonary and respiratory complications and not due to a halt in the natural progression of the disease [18]. Therefore, this expanding patient population currently represents a challenge for the medical community, as patients will gradually require more elaborate and multidisciplinary approaches to treatment. A surveillance and management plan adhering to international standards of care should be carefully implemented and closely monitored to maximize the patient's quality of life [19].

1.2. Genetics and pathophysiology

DMD is caused by mutations in the largest known human gene called dystrophin (*DMD*), which spans 24 kbs of genomic DNA with its 79 exons [20,21]. *DMD* encodes dystrophin, a 427 kDa protein localized on the cytoplasmic side of the sarcolemma of skeletal and cardiac muscle fibers as well as cortical/cerebellar synapses [22–24]. Most common mutations are non-randomly distributed deletions (approximately 68%) that may span one or more exons [25–27]. Surprisingly, the extent of the gene deletion does not correlate with the onset or severity of the clinical manifestations [28]. Exonic duplications, missense, frameshift, point or intronic mutations account for the rest of DMD cases [29–32]. Most mutations result in a shift of the open reading frame and generation of premature termination codons leading to exclusion of one or more exons [33]. These aberrant mRNA transcripts undergo nonsense-mediated decay and therefore almost no dystrophin is produced [34]. Because dystrophin normally functions as an anchor between the actin cytoskeleton and the connective tissue via a sarcoplasmic complex, called the dystrophin glyco-protein complex (DGC) (Fig. 1), muscle fibers that lack dystrophin are subjected to increased mechanical stress and are more susceptible to damage upon contraction [27,35]. Disruption of sarcolemmal integrity causes abnormal influx of Ca^{2+} ions into the cytosol and aberrant activation of calcium binding elements, calcium-dependent proteases and pro-inflammatory cytokines [36]. The above effects further promote skeletal muscle regeneration, as a compensatory action to counterbalance the loss of function [37,38]. The altered myogenic signalling causes impaired proliferative capacity exhaustion of the satellite cell pool and replacement of muscle with fibrotic tissue, eventually resulting in necrosis and muscle wasting [39,40]. This effect is further exacerbated by functional ischemia in the affected muscles, due to the detachment of neuronal nitric oxide synthase from the sarcolemma, where it normally regulates vasoconstriction during muscle exercise [41–43].

2. Exon skipping as a therapeutic strategy for DMD

In the less severe allylic form of DMD, called Becker muscular dystrophy, the generated dystrophin reading frame is maintained in approximately 92% of cases and the generated dystrophin protein product is shorter and less abundant but still partially functional [25]. In Becker patients DMD mutations consist of 70% large deletions, 15% duplications and 15% point mutations and dystrophin detection ranging from 3-10% (severe cases) to even 20% (mild cases) [25,44]. Becker patients may remain ambulatory past 15 years of age and have a milder disease progression attributed to the existing functional dystrophin detected in their muscle fibers. This notion has led to the hypothesis that, if correction of the reading frame in DMD patients is successful, it would lead to the production of a truncated albeit semi-functional dystrophin transcript coding for a protein isoform that will remain resistant to proteolytic degradation and could be properly localized to the sarcolemma [45,46]. In this way, the connection between extracellular matrix and cytoskeletal muscle fibers (costamere) could be restored [47] re-establishing the link to the contractile apparatus [48]. Therefore modification of dystrophin pre-mRNA processing that results to the production of an internally deleted protein while preserving the N- and C- terminal domains which link to the cytoskeleton and extracellular matrix respectively might be the key to DMD future gene therapy [49].

The restoration of the disrupted open reading frame for DMD transcripts that generates the BMD phenotype was originally attempted *in vitro* by targeting exon 19 in the Kobe DMD phenotype [50–52], has now become the basis of the exon skipping therapeutic approach [53]. The strategy uses synthetic single-stranded DNA-like molecules called antisense oligonucleotides (ASOs) that have the potential to hybridize to RNA sequence motifs and to prevent assembly of the spliceosome, restoring the translatable mRNA transcript [54,55]. ASOs are commonly designed to bind to 5' or 3' splice junction and to sterically block access of splicing factors to the target site, altering pre-mRNA splicing [56]. ASOs may also bind to an exonic splicing enhancer or silencer to either promote or block the splicing effect [57].

The unmodified ASOs used originally were subject to degradation by endonucleases and/or exonucleases, therefore, their therapeutic potential was *de facto* very limited [58]. Various chemical modifications of the phosphoribose backbone were performed in order to improve stability, efficacy and pharmacokinetics of ASOs. Substitution of the non-bridging phosphate oxygen with a sulphur atom within the phosphodiester linkage generates the phosphorothioate backbone (PS), which confers increased binding to plasma proteins and resistance to nuclease activity, prolonging the half-life of the ASO [59,60]. In addition, in order to allow for a high-affinity interaction with the target mRNA during splicing to occur rather than mediating mRNA destruction, ASOs should not

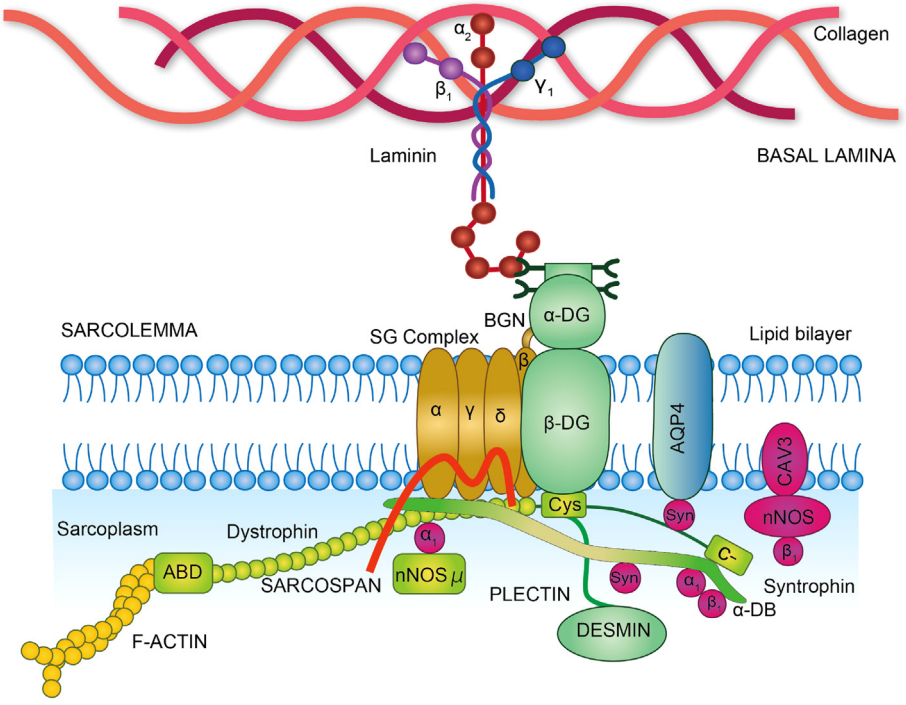

Fig. 1. Extracellular, membrane and cytoplasmatic components of the DGC. The muscle-specific laminin located in extracellular matrix is composed of α2, β1, and γ1 chains. The α2 subunit directly interacts with glycosylated α-dystroglycan (α-DG), which in turn interacts with the transmembrane β-dystroglycan (β-DG). Dystrophin binds to β-DG through cysteine-rich domain (Cys). The transmembrane protein family sarcoglycans (SG) (alpha, beta, gamma and delta) connect the cytoskeleton to the extracellular matrix, conferring structural stability to the sarcolemma. The four subunits of the SG complex interact with each other and with the transmembrane protein sarcospan. The small leucine-rich repeat proteoglycan biglycan (BGN) in the extracellular binds to α- and γ-SG and α-DG. The N- terminal of dystrophin protein (actin binding domain: ABD) binds to F-actin of the cytoskeleton and the C-terminal domain binds to alpha dystrobrevin (α-DB) and syntrophins (Syn). α1 and β1 in dark pink denote α1- and β1-syntrophin, respectively. Aquaporin 4 (AQP4) water channel protein along with syntrophin alpha regulates the efficiency of water transport in myofibers. The cytolinker protein plectin binds β-DG and dystrophin and connects desmin with the DGC. Syntrophins bind directly to α-DB and dystrophin and caveolin 3 (CAV3) through neuronal nitric oxide synthase (nNOS) whereas α1 syntrophin binds to the splice variant of nNOS in skeletal muscle termed nNOSμ.

support ribonuclease H activity [59,61]. Several ASOs fulfilling the above criteria have been tested in cells such as 2′-O-methyl (2′-OMe) and 2′-O-methoxyethyl (2′-MOE) with PS modifications (second generation ASOs) and Locked Nucleic Acids (LNA), ethylene-bridged nucleic acids (ENA), peptide nucleic acids (PNA), tricyclo-DNAs, phosphorodiamidate morpholinos (PMO) (third generation ASOs) [62] (Fig. 2). The most

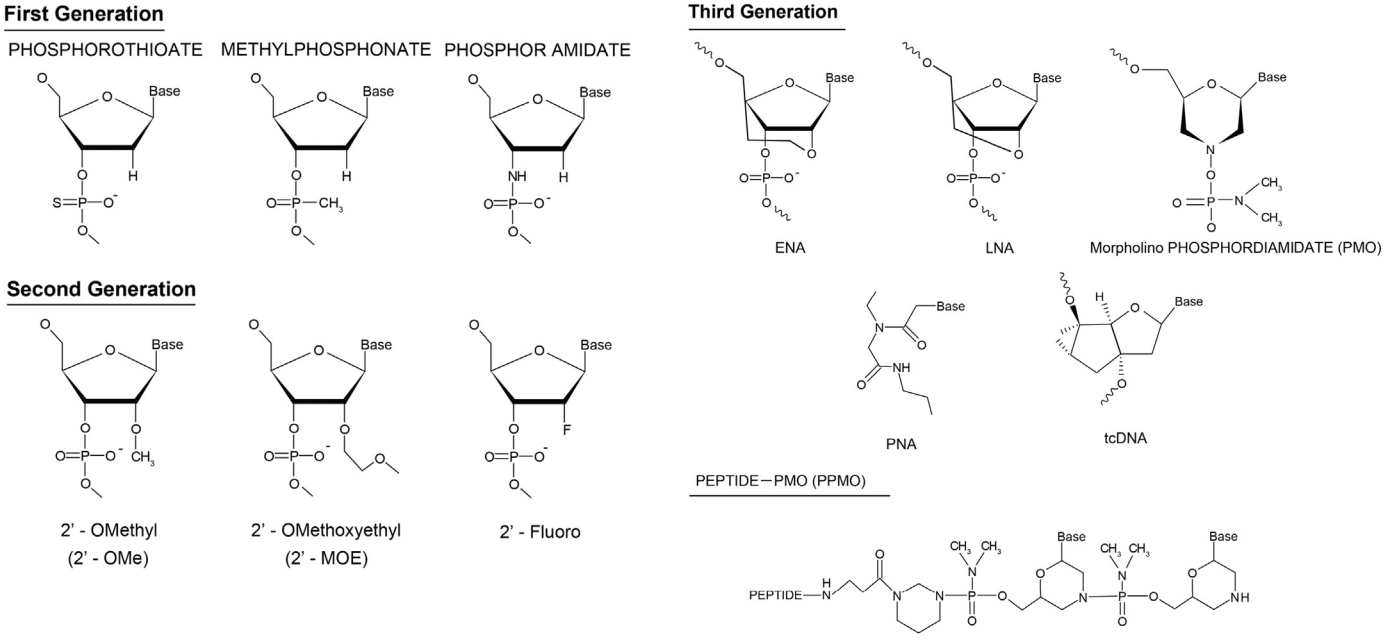

Fig. 2. Chemical structures of first, second and third generation ASOs in comparison to PPMO. Modifications to the phosphodiester backbone of ASOs yielded several analogues such as phosphothioate, methylphosphonate and phosphoramidate that comprise the 1st generation of ASOs. Modifications to the deoxyribose sugar in ASOs yielded compounds such as 2'-OMe, 2'-MOE and 2'-fluoro that belong to the 2nd generation of ASOs. Third generation ASO modifications in ENA, LNA, PNA, tcDNA and PMO confer resistance to nuclease degradation as well as improve binding affinity of compounds. Peptide conjugated PMOs derive from peptide conjugation at the 5' (as shown here) or 3' end of a PMO.

promising ASOs first tested in clinical trials are the 2'-OMe, that consist of methyl modifications to the 2′ position of the sugar moiety and the PMOs [63]. 2′-OMe and 2′-MOE contain a PS backbone and 2′-O-substituted oligoribonucleotide segments. Both 2′-OMe and 2′-MOE ASOs exhibit high nuclease resistance, reduced immune stimulation due to their PS backbone, and are less toxic however they have lower affinity for their target compared to all other modified ASOs [64]. In PMOs, a third generation ASOs, the deoxyribose moiety is substituted by a morpholine ring while the charged phosphodiester inter-subunit linkage is replaced by a non-ionic phosphorodiamidate linkage [65]. This non-ribose based modification renders PMOs immune to nuclease activity but their non-ionic nature minimizes their nuclear uptake [66].

Numerous research groups have shown successful restoration of dystrophin using 2′-OMe and PMO based ASOs in DMD animal models [67]. The most commonly used *mdx* mouse harbours a spontaneous nonsense mutation in exon 23 which results in early termination codon and mild to moderate DMD histopathological features. On the contrary, the more humanized *mdx52* model was generated via targeted deletion of exon 52 which corresponds to the so-called hot spot exon 45-55 region where most DMD mutations are mapped in patients [68,69]. In the *mdx* mouse, exon 23 removal does not disrupt the *DMD* reading frame, allowing for mRNA induction and a little shorter dystrophin production that simulates the Becker phenotype [70]. Intramuscular injection of 1 μg of 2′-OMe ASO administered weekly over a 4 week period or even as a single dose (5 μg) in *mdx* muscle induced dystrophin synthesis and improved functionality of treated muscle [71,72]. Repeated systemic administration of a 2′-OMe ASO elevated dystrophin up to 5% of normal wild type levels in gastrocnemius, intercostal, and abdominal muscles and 1% in quadriceps [73]. Intramuscular or intravenous injections of PMO in *mdx* mice induced body-wide distribution of dystrophin with meaningful therapeutic levels compared to the 2′-OMe ASO studies, albeit with high variability among samples [74,75]. In the *mdx52* mouse model, exon 51 skipping was predicted to generate the Becker phenotype similar to humans. Systemic delivery of a PMO cocktail in the *mdx52* mice seven times at weekly intervals induced 20-30% of wild-type dystrophin expression in muscle, a treatment that could theoretically apply to a high percentage of DMD patients [76,77]. Multi-exon skipping performed on the *mdx52* mutation hot spot (exons 45-55) was achieved by systemic injections of ten PMOs restoring dystrophin levels up to 15%, accounting for over 60% of patients that harbour deletion mutations [78]. Because the *mdx* and *mdx52* dystrophic phenotype is less severe than the one observed in humans, with mildly impaired muscle function and normal lifespan due to the utrophin compensation, there is a need to find alternative animal models to test efficacy, pharmacodynamics and safety of the drug [79,80]. For this purpose, multi exon skipping (exon 6-8 or exon 6-9) using PMO cocktail was successfully applied in the golden retriever canine dog model *in vitro* [81] and in canine X linked muscular dystrophy model $CXMD_J$ *in vivo* [82] where it ameliorated pathological phenotype without triggering any serious side effect. Collaborative projects using the $CXMD_J$ model bred in our facilities have thoroughly investigated the systemic efficacy and safety of 6-9 multi-exon skipping using combinations of PMO cocktails [82–84] and a similar approach has been adopted to our current studies that assess the potency and safety of novel PPMO cocktails (Fig. 3).

3. ASOs in exon skipping clinical trials targeting DMD

The majority of *DMD* mutations in humans cluster between exons 45-55 the so-called 'hot spot' and mutations in exon 51 represent 13% of the DMD patient population, making this subgroup the most attractive target for clinical trials using the single exon skipping approach [85]. The first experimental clinical trial used 2'-OMe-PS based drug called drisapersen (PRO051/GSK2402968/Kyndrisa) targeting exon 51 was developed by Prosensa, GlaxoSmithKline (GSK) and lately BioMarin. Preliminary data from phase I clinical trial was very encouraging, indicating dystrophin build up in a dose-dependent manner reaching up to 15.6% of that of healthy muscle [86]. Two randomized placebo-controlled phase 2 clinical trials demonstrated improvement of the 6-minute walk test (6MWT) in children treated with 6 mg/kg/week drisapersen administered subcutaneously for a period of 24 weeks the effect which was maintained, albeit with reduced significance, 48 weeks after treatment [87]. However results were not reproducible in the phase III placebo-controlled trial that followed and no dystrophin production was detectable by western blotting in treated patient's muscle obtained through biopsy [88,89] although an increase in sarcolemmal dystrophin myofiber was observed by immunohistochemistry [90–92]. Adverse effects such as skin fragility at the site of injection, proteinuria and presence of alpha microalbumin in the urine occurred and became more prominent when dose was scaled up to 9 mg/kg/week, therefore, the US Food and Drug Administration (FDA) declined approval on the basis that 'the standards of effectiveness have not been met' [90]. However, intensified efforts to overcome such toxicity problems have led to the development of optimized stereopure ASOs. A characteristic example is WVE-210201, designed to skip exon 51 in the *DMD* gene, which has yielded very promising *in vitro* and mouse-based exon skipping data and its efficacy and safety are currently assessed in a phase 1 clinical trial [93,94].

The FDA recently approved eteplirsen (Exondys 51, AVI4658; Sarepta Therapeutics), targeting exon 51 in DMD patients, which achieved around 42% positive dystrophic fibers when administered intramuscularly to the extensor digitorum brevis in DMD patients in a single-blind placebo-controlled trial [90–92]. Therefore eteplirsen, like drisapersen could successfully mediate 51 exon skipping in dystrophic patients [86,92,95,96]. Subsequently, eteplirsen was tested systemically in a non-randomized (phase I/II) [92] and randomized (phase II) clinical

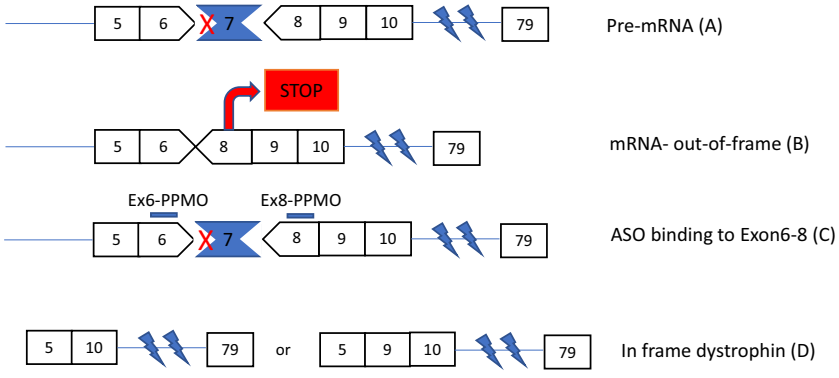

Fig. 3. Exon skipping strategy in $CXMD_J$ dog model using PPMOs. A point mutation in exon 6 is responsible for the loss of exon 7 in dystrophic $CXMD_J$ dog (A) ultimately resulting to out of frame mRNA (B) and disruption of dystrophin protein production. PPMOs sequences manufactured in such a way to bind in exon 6 and 8 (C) cause effective splicing of either exon 6-7-8 or 6-7-8-9, restoring the dystrophin reading frame.

trial [91] showing skipping of exon 51 of variable nature in all patients and substantial increase in dystrophin-positive fibers in the high dose cohort groups or longer treated cohort groups with minimal side effects. Further functional assessments using the 6MWT demonstrated a decreased rate of ambulation loss in eteplirsen treated patients [97]. However more elaborate assessments indicated that variability in exon skipping efficiency occurred among patients and dystrophin was restored non- homogenously between different muscles, limiting the drug's therapeutic potential [98]. Currently, the success in exon skipping therapy is determined by the percentage efficiency of exon skipping and the resulting expression level of the restored protein but these two parameters are not always well correlated and may not account for the observed phenotype or strongly deviate from earlier laboratory findings [99]. For example, in eteplirsen-treated patients, 30-50 mg/kg doses administered weekly 48 weeks resulted in a mean of 47.3% of dystrophin-positive fibres in original clinical trials, but when the percentage of dystrophin protein for the same patients was re-evaluated by FDA using the western blot technique which is considered a more reliable quantification method, it was as low as 0.93% compared to 0.08% in untreated controls [85,91]. Furthermore, systemic administration of eteplirsen appeared to increase dystrophin positive fibres up to 23% in phase I trials, but the latest data show that eteplirsen may only restore dystrophin up to 0.28%, yielding similar findings with the non-approved drisapersen [97,100,101]. Taking into account that mild dystrophinopathy is observed in patients that possess 10-25% of dystrophin we can make the assumption that achieving at least 10% of *DMD* mRNA knock up is essential for ameliorating the DMD phenotype [102,103] whereas 4% of dystrophin may be enough to significantly increase survival in severely diseased patients [104]. Both percentage targets mentioned above are far from the ones highlighted by the statistics obtained in the eteplirsen's trials [105]. Furthermore, whether an elevation in the percentage of dystrophin-positive fibres is significant and can be deemed responsible for the amelioration of the clinical performance of a patient as measured by 6MWT is debatable, especially after pooling statistics in eteplirsen's case [106].

While approval of eteplirsen was non unanimous and controversial, sparking a debate among FDA members and scientists and it is still not conclusive whether eteplirsen can successfully halt disease progression in diagnosed DMD patients [89,107,108], this decision will hopefully pave the way for more elaborate and robust PMO clinical trial designs. Recently, in our institute we concluded a phase I open-label study in collaboration with Nippon Shinyaku Co. Ltd., dose-escalation clinical trial to evaluate exon 53 skipping efficacy of NS-065/NCNP-01 PMO which could be targeting 8% of DMD patient population [53]. NS-065/NCNP-01 PMO demonstrated dose-dependent exon skipping efficacy and successful dystrophin expression with minimal side effects [109]. Recently released data by Nippon Shinyaku indicate that the level of dystrophin expression in 8 patients participating in Phase I/II clinical trial averaged 5.21%, well-surpassing efficacy data reported with the approved drug eteplirsen. Sarepta Therapeutics has recently announced its plan to submit a new drug application for obtaining accelerated approval of the exon 53 targeting PMO based drug called golodirsen (SRP-4053) after generating promising data in a phase I/II clinical trials [110]. The efficacy and safety of golodirsen and of another PMO drug, called casimersen (SRP-4503), which targets exon 45 of the *DMD* gene (approximately corresponding to 8% of patient population) are currently evaluated in the ongoing ESSENCE phase III double-blinded assessment (NCT02500381). This new era of ASO based drugs should lead to novel treatment regimens that will succeed in delivering dystrophin restoration with minimal adverse effects, bringing hope to patients suffering from genetic neuromuscular diseases.

4. PMO limitations and the development of PPMOs

Some of the main limitations of PMOs as a therapeutic agent for DMD underlined by numerous animal-based studies are: poor cellular uptake and permeability of membrane barriers, rapid clearance from systemic circulation, inability to cross blood-brain barriers, variability of dystrophin expression and distribution in various target tissues or within the same tissue, short duration of the exon skipping effect requiring repetitive administration and/or high dosage of the drug [45]. Several of the mouse-based studies conducted aimed to ameliorate the poor PMO uptake in systemic administration and enhance intracellular delivery, but little progress has been made so far [73–75,111,112]. In fact, high and repeated systemic doses of PMOs are necessary to achieve upregulation of dystrophin in skeletal muscles in animal models [82,113] without any significant effect in diaphragm or heart. These observations are not unexpected when considering PMO's chemical nature. Originally PMOs were thought as molecules that have no net electrical charge and thus are unable to form complexes with delivery vectors, a fact that minimizes their off-target effects and renders them suitable for intramuscular administration but unfortunately reduces their overall ability to cross cell membranes and thus their systemic efficacy [114]. Based on research data very recently published by our group, PMO may have negative zeta potential which enables them to form complexes [115]. Because PMO uptake can occur by passive diffusion, the dystrophin-deficient leaky muscle fibers can more readily internalize intramuscularly administered PMOs [77,116]. In fact, eteplirsen has the potential to penetrate leaky muscle cells to exert its therapeutic effect however once the treated muscle starts building up dystrophin, it automatically becomes less leaky and thus less penetrable preventing additional entry of PMO and hampering an homogeneous dystrophin build up [98]. This notion is in agreement with interpretations of data derived from PMO trials using the *mdx* mouse model. It was observed that cycles of muscle regeneration/degeneration had taken place during an intermittent PMO systemic high dose delivery scheme, a fact which can possibly account for the dystrophin fluctuations observed among treated mice and the inability of the delivered dosage to protect dystrophic muscle from the eccentric contraction-induced damage [117].

On the other hand, PMOs are almost always successfully endocytosed but due to the hydrophobicity of the plasma membrane, only traces of internalized PMOs can escape endosomes and reach their target [118]. Therefore, delivery of PMOs in tissue culture is aided by the use of endo-porter which is converted to its poly cationic form inside the acidified endosomal compartment, rendering the endosomal membrane permeable [119]. Covalent linkage of an octaguanidinium dendrimer scaffold on the 3' end of a PMO ring leads to the generation of a modified morpholino called *vivo* morpholino (vPMO) [120]. vPMOs have been tested in the *mdx52* mouse model as well as dystrophic dog models, and in both cases, they have shown optimized efficiency in splicing modulation and skeletal dystrophin production [121–123].

An effective way to enhance PMO penetration in cell membrane is to conjugate them to short cell penetrating peptides (CPPs). CPPs, also known as protein transduction domains are short peptides of cationic, amphipathic or hydrophobic nature that have the ability to form a complex with cargo molecules and successfully transport active biological conjugates inside the cell [124,125]. Experiments using such CPPs including HIV-1 Tat protein [126,127], Drosophila antennapedia protein and oligoarginine peptides as crosslinkers enhanced PMO uptake in cells [128]. Internalization of HIV-1 Tat protein conjugates can be impaired possibly due to the strong electrostatic interactions that are formed with cellular heparin sulfates during endocytosis [129]. The third alpha helix domain of antennapedia also known as penetratin, is widely used to maximize the efficiency of internalization, but fails to deliver any significant amounts to the nucleus [120]. Moreover, enzyme degradation throughout delivery and instability of the above conjugates in human serum are factors that pose hurdles towards their use in systemic delivery and thus limit their therapeutic potential. To enhance PMO delivery, CPPs were subsequently enriched with arginines, because as cationic amino acids they could potentially facilitate delivery

of the neutrally charged PMOs into cell compartments [130,131]. Conjugation of PMOs to a penetratin which contains six arginines residues near the N-terminus (R_6-Penetratin) and a bulky side-chain composed of hydrophobic amino acids generated a new class of PMOs called PMO internalization peptides (Pips) with enhanced cellular uptake capacity and stability against serum proteolysis [63,132,133]. Insertions of 6-aminohexanoic acid residues (X) into an R_8 peptide increased the corresponding CPP's serum stability and nuclear delivery but failed to prevent intracellular degradation. Incorporation of non-α-amino acids into the oligoarginine (R_8) peptides prevented potential endosomal entrapment and thus greatly enhanced their metabolic stability [134] whereas insertion of β-alanines into this skeleton further increased intracellular stability [134–136].

Originally, CPPs used for conjugation derived from naturally occurring proteins already proven to have excellent translocation properties [137], but further understanding of the structural activity relationship of CPPs led to synthesis of CPPs based on predictive algorithms [138] therefore existing CPPs are heterogeneous in nature. The resulting peptide conjugated morpholinos (PPMOs) are taken up *in vitro* by proliferating myoblasts or terminally differentiated myotubes via a poorly understood process called gymnosis [139], that does not require any vehicle or transfecting reagent for delivery [140–143]. In fact, arginine-rich CPP conjugation to PMO not only remarkably enhanced cellular uptake of PMOs but also improved their pharmacophore potency as described in detail in later sections. For a summary of *in vitro* and animal studies undertaken in the field of DMD, please refer to table 1.

5. Comparison of PPMO and PMO properties

5.1. Improved internalization into cells

Internalization of PMOs into cells can occur via interchangeable pathways which are dependent upon the nature of the cell (Fig. 4) [144,145]. Cell surface PMO adsorption is mediated by a clathrin and caveolin-dependent or less frequently independent endocytic processes [146] and aided by cell surface receptors such as integrins, G protein-coupled receptors, receptor tyrosine kinase, Toll-like receptors and scavenger receptors [61]. Once ASOs are internalized in cells *via* endocytosis, they have to effectively be delivered to the nucleus to exert their splicing effect. CPP conjugation is implemented in order to increase cellular uptake of PMOs, which is very poor and requires large dosage and repeated administration to reach their target [147]. Similarly to PMO uptake, the most prevalent theory regarding PPMO uptake is *via* endocytosis mainly mediated by class A scavenger receptor subtypes (SCARAs) [141,148]. This interaction is greatly enhanced by the propensity of amphipathic PPMOs to self-assemble into nanoparticles [141]. Furthermore, the net charge of PPMOs, called zeta potential, is negative when measured in isotonic media and may influence the interaction of individual PPMOs with the plasma membrane, strengthening the notion that a receptor is necessary for internalization of PPMOs [137]. Addition of penetratin boosted the ability of PPMO to penetrate the cell membrane of differentiated neuronal cultures [149]. Addition of a B peptide has shown to facilitate heparin sulphate proteoglycan binding, thus enhancing internalization of PPMO to the endosomal pathway [114,150]. It was observed that Pip6-PMO was more readily internalized by H2K-*mdx52* and C2C12 myotubes rather than myoblasts possibly due to higher endosomal entrapment that limits its availability and this was independent of the leakiness of the membrane [151]. The same group attributed the diminished potency of Pip6-PMO in cardiac cells versus skeletal muscle cells to different endocytic pathway internalization routes.

5.2. Enhanced potency at lower doses

A considerable hurdle in PMO endocytosis which contributes to their limited systemic therapeutic effect is their endosomal entrapment [152,153]. Peptides containing polyarginine analogues can induce leakage of endosomes and aid in the release of the conjugated PMOs into the cytosol [154]. PMOs and first-generation PPMOs that had a Tat or penetratin backbone required high concentrations of the compound to induce efficient and targeted exon skipping as well as to escape endosomal entrapment. Addition of R6 penetratin in the Pip compounds allowed efficient splicing at much lower doses [133,136]. In fact, injection of Pip2a or Pip2b conjugated PPMOs in the tibialis anterior of the *mdx* mouse resulted in efficient exon 23 skipping and significantly higher dystrophin rescue compared to the naked PMOs [132]. Pre-treatment of tibialis anterior muscle of *mdx* mice with PPMOs allows for a rescue of dystrophin expression at low dosages of AAV administration by prevention of AAV genomic loss, potentiating the microdystrophin based gene therapy [155].

Repeated intraperitoneal administration of a PPMO in the double utrophin/dystrophin KO mouse which shows a more severe phenotype than the *mdx* mouse and is deemed a more appropriate model to test the therapeutic effect of PMO [156] restored dystrophin expression in most skeletal muscles including diaphragm and prevented the onset of the dystrophic phenotype [157]. However, PPMO administration at a more advanced stage of disease failed to prevent disease progression although significantly delayed the disease progression when applied to mice in an early stage of disease [158].

5.3. Sustained dystrophin production

Evaluation of a series of PPMOs comprising a variable number of 6-aminohexanoic acid (X) and β-alanine (B) residues through intraperitoneal delivery in EGFP-645 mice that use the EGFP-654 pre-mRNA reporter to ascertain PPMO entry to cells. It was shown that the B conjugated peptide was the most effective one in sustaining dystrophic protein expression and targeting heart, diaphragm and quadriceps, key muscles in DMD patients [159]. Independent studies on B-PMO conjugated administered by intravenous injection to *mdx* mice have confirmed the high efficacy of B-PMO in dystrophin correction in *mdx* skeletal muscle with no overt hepatic or renal toxicity observed [160]. Direct demonstration that cell penetrating peptides may accentuate *in vivo* nucleic acid delivery came by the same group one year later. The authors describe how a chimeric fusion peptide generated by conjugation of a muscle-specific heptapeptide and a B peptide can induce effective exon skipping resulting in an efficient restoration of dystrophin in multiple skeletal muscles as well as significant increase in muscle strength in the *mdx* mouse model [161].

5.4. Improved efficiency of systemic delivery to target tissues

The advantage of PPMOs over PMOs to induce efficient systemic and target specific exon skipping and ameliorate the DMD phenotype was evident from the pioneering studies in *mdx* mice [121,159–163] and has been confirmed by virtually every study undertaken since then [147,164]. The generation of peptide nucleic acids/ PMO internalization peptides (Pips) series which contain two arginine-rich domains separated by a central short hydrophobic core were designed in order to improve serum stability and drive efficient exon skipping in a variety of target tissues, increasing heart dystrophin production [132,133]. Indeed, both Pip5 and Pip6 PMO series were capable of restoring dystrophin expression body wide following a single intravenous injection [150,165]. Further studies on Pip6 series demonstrated amelioration of DMD pathology and phenotype in exercised mice [151,166]. Dystrophin built up in more tissues aided restoration of DGC integrity with proper localization of beta-dystroglycan and improvement in muscle power and improvement of the phenotype in mice [161] and dogs [167]. Identification of candidate muscle-homing peptides and subsequent conjugation to ASOs, may improve delivery to target tissues, maximizing therapeutic effects [168,169].

Table 1
List of developed PPMOs and their therapeutic effects in experimental models.

Compound name	Sequence	System	Route of administration	Age begin treatment	Dosage regime	Dystrophin restoration	Ref
B-peptide based	(RXRRBR)2XB	$CXMD_J$	im	4–5 mo	3,600 μg/1,200 μg	skeletal muscle	166
			single ic/iv	5 mo	12 mg/kg	skeletal and cardiac muscle	
			systemic iv	4–5 mo	12 mg/kg	skeletal and cardiac muscle	
Pip6a-PMO	RXRRBRRXR YQFLI RXRBRXRB	mdx	im pretreatment	1 nmole	1 nmole	enhances DMD rescue by AAV	155
B-PMO	$(RXRRBR)_2XB$	mdx	ip	21 wk	19 mg/kg dose	diaphragm	166
			iv	21 wk	19 mg/kg dose	diaphragm, intercostal, sternomastoid	
M12	RRQPPRSISSHP	mdx	iv 3x weekly	6–8 wk	25 mg/kg	skeletal muscles	163
			iv single	6–8 wk	75 mg/kg	skeletal muscles	
PMOE23	(RXRRBR)2XB	DKO	iv biweekly	20–29, 30–39, 40–49, 50+ days	15 mg/kg	early treatment prevents onset	158
Pip6a	RXRRBRRXR YQFLI RXRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	diaphragm,skeletal and cardiac (high)	
Pip6b	RXRRBRRXR IQFLI RXRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	diaphragm (high),skeletal and cardiac	
Pip6c	RXRRBRRXR QFLI RXRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	diaphragm,skeletal and cardiac	
Pip6d	RXRRBRRXR QFL RXRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	diaphragm,skeletal and cardiac	
Pip6e	RXRRBRRX YRFLI RXRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	diaphragm,skeletal	
Pip6f	RXRRBRRXR FQILY RXRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	diaphragm,skeletal and cardiac	
Pip6g	RXRRBRRX YRFRLI XRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	low	
Pip6h	RXRRBRRX ILFRY RXRBRXRB	H2K-mdx	*in vitro*		0.125 – 1 μmol/L	exon skipping observed	165
		mdx	iv	4 to 5 mo	12,5 mg	low	
B-peptide based	(RXRRBR)2XB	mdx	iv	4 to 5 wk	30 mg/kg	20-50% in skeletal muscle	72
			iv biweekly/year		1.5 mg/kg	low	
			iv biweekly/year		6 mg/kg	skeletal muscles and heart	
			iv monthly/year		30 mg/kg	skeletal, diaphragm	
Pip5e	RXRRBRRXR-ILFQY-RXRBRXRB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	highest TA restoration	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	diaphragm, heart and skeletal	
Pip5f	RXRRBRRXR-ILFQY-RXRXRXRB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	dystrophin observed	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	
Pip5h	RXRRXR-ILFQY-RXRRXR	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	dystrophin observed	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	
Pip5j	RBRRXRRBR-ILFQY-RBRXRBRB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	highest TA restoration	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	
Pip5k	RBRRXRRBR-ILFQY-RXRBRXRB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	dystrophin observed	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	
Pip5l	RBRRXRRBR-ILFQY-RXRRXRB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	dystrophin observed	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	
Pip5m	RBRRXRRBR-ILFQY-RXRBRXB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	dystrophin observed	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	
Pip5n	RXRRBRRXR-ILFQY-RXRRXRB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im	2/ 6 mo	5 μg/kg	highest TA restoration	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	

Table 1 (*continued*)

Compound name	Sequence	System	Route of administration	Age begin treatment	Dosage regime	Dystrophin restoration	Ref
Pip5o	RXRRBRRXR-ILFQY-RXRBRXB	H2K-mdx	*in vitro*		1, 2 μmol/l	exon skipping observed	150
		mdx	im		5 ug/kg	dystrophin observed	
		mdx	iv single	2/ 6 mo	25, 18.75, 12.5 mg/kg	heart and skeletal	
P007	$(RXR)_4XB$	DKO	ip weekly x 6	10 days	25 mg/kg/week	skeletal muscle, diaphragm	175
B peptide	$(RXRRBR)_2XB$	mdx	iv single	6 mo	18.75 mg/kg	skeletal muscles	
PMO-Pep	(RXR)4XB	DKO	ip x 6	10 days	25 mg/kg/week	skeletal muscles, prevents onset	157
B-PMO	RXRRBRRXRRBRXB	mdx	iv x 4 dailyx2 wk	8/16 wk	12 mg/kg/day	skeletal, cardiac, smooth muscles	185
B-PMO	RXRRBRRXRRBRXB	mdx	iv	6-8 wk	25 mg/kg	skeletal muscles, heart	161
			iv X 6 weekly	6-8 wk	3 mg/kg/wk	TA, quadriceps	
			iv X 3 weekly	6-8 wk	6 mg/kg/wk	skeletal muscles	
MSP-PMO	ASSLNIAXB	mdx	iv	6-8 wk	25 mg/kg	TA and quadriceps	161
B-MSP-PMO	RXRRBRRXRRBRXB-ASSLNIAXB	mdx	iv x 6 weekly	6-8 wk	3 mg/kg/wk	TA and quadriceps	161
MSP - B*-PMO	ASSLNIAXB-RXRRBRRXRRBRXB	mdx	iv x 6 weekly	6-8 wk	6 mg/kg/wk	TA only	161
P007	$(RXR)_4XB$	mdx	iv	6-8 wk	25 mg/kg	heart, biceps, diaphragm	160
			iv x 3		6 mg/kg/wk	skeletal muscles and heart	
B peptide	$(RXRRBR)_2XB$	mdx	iv	6-8 wk	25 mg/kg	skeletal, lower than P007	160
			iv x 3		6 mg/kg/wk	skeletal muscles, lower efficiency	
J-PMO	(rXr)4XB	EGFP-654	ip x 4	7–8-wk	12 mg/kg	quadriceps	159
M23D-B	RXRRBRRXRRBRXB	mdx	sc x 4	7–8-wk	12 mg/kg	cardiac muscle, diaphragm,	159
			iv x 4	7–8-wk	12 mg/kg	quadriceps	
			ip x 4	7–8-wk	12 mg/kg		
PPMOE23	RXRRBRRXRRBRXB	mdx	im	4–5 wk	2 μg	TA	162
			iv	adult	30 mg/kg	diaphragm, skeletal, cardiac muscle	
			iv x 6 biweekly	adult	30 mg/kg	diaphragm, skeletal, cardiac muscle	
Pip1	RXRRXRRXR IKILFQN RRMKWKK	H2K mdx	*in vitro*		1 or 2 μM	efficient exon23 skipping	132
Pip2a	RXRRXRRXR IdKILFQNd RRMKWHKB	mdx	im	6-8 wk	5 mg	dystrophin restoration in TA	
Pip2b	RXRRXRRXR IHILFQNd RRMKWHKB	mdx	im	6-8 wk	5 mg	dystrophin restoration in TA	
MSP	ASSLNIA	H2K mdx	*in vitro*		250 nmol/L	exon skipping observed	127
		mdx	im	2 mo	5, 10, and 20 μg	dystrophin upregulation in TA	
		mdx	im	3 wk, 6 mo	5 μg	dystrophin upregulation in TA	
TAT	YGRKKRRQRRRP	H2K mdx	*in vitro*		250 nmol/L	exon skipping observed	127
		mdx	im	2 mo	5, 10, and 20 μg	dystrophin upregulation in TA	
		mdx	im	3 wk, 6 mo	5 μg	dystrophin upregulation in TA	
AAV6	TVAVNLQSSSTDPATGDVHVM	H2K mdx	*in vitro*		250 nmol/L	exon skipping observed	127
		mdx	im	2 mo	5, 10, and 20 μg	dystrophin upregulation in TA	
		mdx	im	3 wk, 6 mo	5 μg	dystrophin upregulation in TA	
AAV8	IVADNLQQQNTAPQIGTVNSQ	H2K mdx	*in vitro*		250 nmol/L	exon skipping observed	127
		mdx	im	2 mo	5, 10, and 20 μg	dystrophin upregulation in TA	
		mdx	im	3 wk, 6 mo	5 μg	dystrophin upregulation in TA	
PMO-Pep	(RXR)4XB	mdx	ip single	neonatal	1,2,5,10,25 mg/kg	diaphragm	173
			ip weekly x 6	neonatal	1,2,5 mg/kg	skeletal muscle, diaphragm	
			ip weekly x 4	neonatal	5 mg/kg	skeletal muscle, diaphragm	

R: arginine, B: beta alanine, X: 6 aminohexanoic acid, wk: week, mo: month, im: intramuscular, iv: intravenously, sc: subcutaneously, ic: intracoronary, ip: intraperitoneal

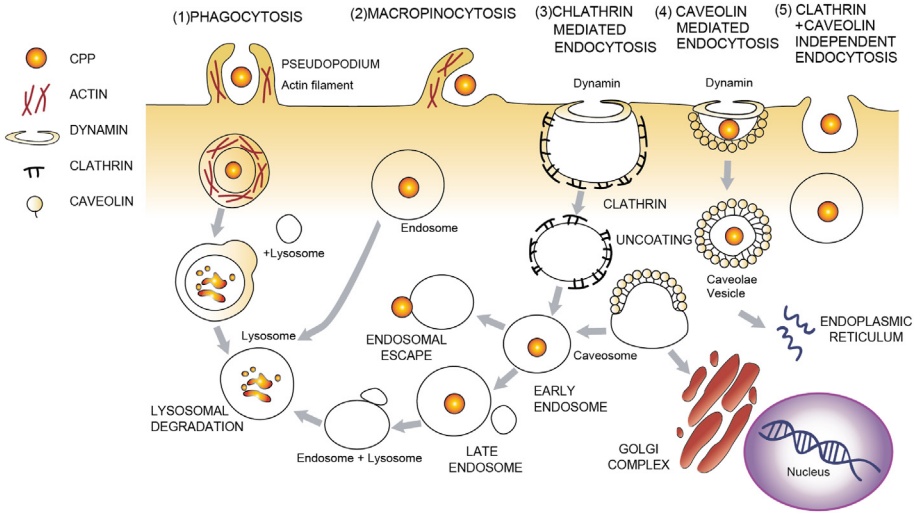

Fig. 4. Cellular internalization of CPPs through various endocytotic pathways. ASOs are adsorbed and internalized in the cell via different routes including 1) Phagocytosis 2) Macropinocytosis 3) Clathrin-mediated endocytosis 4) Caveolin- mediated endocytosis and 5) Clathrin/caveolin- independent endocytosis. Once internalized ASOs may traffic from early endosomes to lysosomes and Golgi. To exert their function, ASOs must be able to escape from endosomes and reach the nucleus.

5.5. Diaphragm targeting

In DMD patients, diaphragm function is severely compromised, leading to a progressive decline of ventilation and premature death [170]. In the *mdx* mouse model, diaphragm exhibits a fibrotic pattern with loss of elasticity and increased collagen density, becoming the best muscle to study representative histological changes of dystrophic phenotype in this animal model [171]. Repetitive administration of an $(R\text{-}X\text{-}R)_4XB$–PMO conjugate (X= aminohexanoic acid and B: β alanine) effectively restored dystrophin expression in the diaphragm of *mdx* mice, when treatment was applied as early as the neonatal stage, however this effect was discontinuous at longer intervals after the final injection [172]. Dystrophin expression in diaphragm of neonatal *mdx* mice increased dose-dependently after a single intraperitoneal injection of 1, 2, 5 and 10 mg/kg of an (RXR)4XB peptide-based PPMO, reaching levels comparable to wild-type mice [173]. Systemic administration of the chimeric B-MSP-PMO at 6 mg/kg induced diaphragmatic dystrophin levels similar to gastrocnemius and biceps ones [174]. Intraperitoneal injection of P007-PMO at a dosage of 25 mg/kg/week for six weeks highly restored diaphragmatic levels of dystrophin and ameliorated the severe pathology of dystrophin/utrophin double knock-out mouse [175]. Studies carried out using the same P007-PMO revealed that a single intravenous administration of 25 mg/kg restored up to 25% of diaphragmatic dystrophin but this percentage dropped when a lower dosage of 5 mg/kg was administered systemically in the *mdx* mice [160]. The Pip5 and Pip6 series improved dystrophin diaphragmatic targeting [150,165]. A single dose of 19 mg/kg of B-PMO administered intraperitoneally markedly restored diaphragmatic dystrophin whereas the same dosage delivered intravenously also elevated dystrophin in intercostal and sternomastoid muscles of *mdx* mice [176].

5.6. Cardiac muscle targeting

Cardiomyopathy is an unavoidable consequence of DMD and is present in almost all patients over 18 years as a form of dilated cardiomyopathy [177] accounting for 20% of the mortality [178]. In DMD patients, absence of myocardial dystrophin leads to fibrosis, conditions that aggravates cardiac workload and stimulates autonomous system to increase heart rate as a compensation mechanism, further worsening the existent ventricular dysfunction [12].

PMO administration failed to aid cardiac function improvement in animal models tested [75], possibly due to inability of the drug to enter the impermeable cardiomyocytic membrane [179]. Even high doses of PMO (60 mg/kg) administered intravenously at biweekly intervals for one year in the *mdx* mouse have shown little efficacy in upregulating dystrophin protein levels in myocardium or in improving cardiac output and stress response [180]. Effective exon 23 skipping was observed in cardiac myoblasts obtained from *mdx* mice in *vitro* albeit with much higher doses than the ones necessary to produce the same effect in skeletal muscle cells [179,181]. Low levels of skipping efficiency in cardiac muscle (range of 2-3%) upon PMO systemic administration has been observed in almost every PMO study published so far, despite achieving high-efficiency dystrophin expression in most of the skeletal muscles [76]. Notably, intra-cardiac injections of naked morpholino oligos in aged *mdx* mice yielded very low exon skipping percentages [182].

Up to now, no available treatment is capable of restoring dystrophin protein in the heart of DMD patients. This fact is of pivotal significance, not only because of the high risk of failure of proper cardiac function *per se* which remains one of the main culprit of premature mortality in DMD patients but also because aggravation of cardiac disease progression occurs as a result of increased work load originating from amelioration of skeletal muscle function and enhanced locomotor activity using the current treatment regime [183,184].

The need for more efficient targeting of in-frame exon skipping particularly in cardiac muscle has led to the exploration of diverse structural PPMOs. Restoration of dystrophin in skeletal muscles and diaphragm in *mdx* mouse and in the more severely affected dystrophin/utrophin double knock out mouse, in absence of cardiac dystrophin expression, restored cardiac function to wild type levels suggesting that targeting respiratory muscles may prevent cardiomyopathy in DMD patients [175]. However intravenous administration of 19 mg/kg dose of B-PMO delivered intravenously or intraperitoneally restored dystrophin level of respiratory muscles but failed to improve cardiac function in *mdx* mice [176]. An arginine-rich PPMO with a backbone of $(RXRRBR)_2XB$- targeting exon 23 in the *mdx* mouse named as PMOE23 successfully restored dystrophin almost to normal levels and at the same time protected heart muscle from damage after dobutamine stress challenge [162]. Systemic administration of the arginine-rich PPMO, AVI-5225 that induces exon 23 skipping, efficiently rescued cardiac dystrophin in the *mdx* mouse and inhibited onset and progression of cardiomyopathy [159,185]. Up to 20% of dystrophin expression was detected in the heart of *mdx* mice, three week after administration of a single intravenous injection of P007-PMO which has a $(RXR)_4XB$- backbone and is more effective than the $(RXRRBR)_2XB$- peptide [160]. It was found that a dosage of 6 mg/kg biweekly for a year rather than the higher treatment regime of 30 mg/kg monthly restores dystrophin in cardiac muscle up to 5%, indicating that treatment spacing is equally important to dosage regime [72].

A single intravenous injection of Pip5e-PMO -conjugated peptide induced 50% dystrophin expression in the heart of *mdx* adult mice, attributed to an increased nuclear delivery of Pip5e-PMO in cardiomyocytes [150]. Generation of the Pip6 series PMO by altering the peptide hydrophobic core sequence of Pip5e-PMO was carried out in order to promote homogeneous dystrophin restoration and particularly to more efficiently target the cardiac muscle. In fact, inversion of the Pip5e-PMO hydrophobic core (Pip6a) yielded cardiac dystrophin recovery score as high as 37% in the *mdx* mouse model whereas the specific arrangement of hydrophobic residues within the core did not alter the efficacy of the construct's exon skipping [165]. The same group has shown that administration of Pip6f-PMO (scrambled peptide core) may restore dystrophin protein levels up to 28% in the heart of *mdx* mice previously subjected to a forced exercise regimen to induce changes that mimic the DMD cardiac phenotype [166]. Moreover, these mice exhibited lower levels of fibrosis, inflammatory and oxidative markers as well as other signs indicative of cardiomyopathy progression.

In the $CXMD_J$ dog model, 4 monthly intravenous injections of the B peptide conjugated PMO cocktail or a single intracoronary or intravenous injection successfully induced 6-9 multi exon skipping and rescued dystrophin expression in most parts of cardiac muscle, as assessed by western blotting [167]. The dystrophin protein expression ameliorated vacuole degeneration in Purkinje fibres and increased Q/R ratio in the treated dogs.

6. The use of PMO and PPMO in neurodegenerative diseases

The term neurodegenerative diseases encompass a range of progressive disorders characterized by the gradual degeneration of the structure and function of the nervous system [186]. Representative examples are Parkinson's disease, Alzheimer's disease, Huntington's disease (HD), Amyotrophic Lateral Sclerosis (ALS), spinal muscular atrophy (SMA) and spinocerebellar ataxias. There is a diverse range in pathophysiology; memory and cognitive functions might be gradually compromised while mobility and speech are unaffected, or the opposite or eventually all functions may be affected [187]. Risk factors and disease severity may be correlated with an advanced age (e.g. Alzheimer's) or genetic predisposition (e.g. HD) or both (e.g. Parkinson's, early onset Alzheimer's) but primary cause for most of the neurodegenerative diseases is yet to be identified. As the percentage of aged population is rapidly expanding worldwide, global efforts to find new cures for neurodegenerative conditions that are linked to changes found in aged brains are intensified [188].

CPPs show improved systemic delivery and cellular uptake and due to their proven transmembrane transporting capacity they have been listed as promising agents in the treatment of central nervous system diseases (CNS). It was assumed that the otherwise impermeable blood-brain barrier (BBB) [189], being a negatively charged membrane formed by endothelial cells, may demonstrate increased affinity for the small size cationic or amphipathic CPPs [190,191] especially if systemic inflammation is present in individuals with abnormal neurological conditions [192]. In cases where CPPs have an arginine core, the high charge density generated may further enhance their BBB influx rates [193]. CPPs can cross the BBB using different transport mechanisms. In adsorptive-mediated transcytosis, the strong electrostatic interactions generated by the negatively charged phospholipids may aid translocation of the CPPs across the hydrophobic core of the membrane [194]. For example such a mechanism was employed in order to deliver the anti-apoptotic protein B-cell lymphoma-extra large (Bcl-xl) fusion protein with Tat to the murine brain as a cure for ischaemic injury [195]. In receptor-mediated transcytosis, interaction of the CPPs with a transporter localized at the endothelial cell surface such as the low-density lipoprotein receptor (LDLR), the low-density lipoprotein receptor-related proteins 1 and 2 (LRP1 and 2), the scavenger receptors class A type I (SR-AI), class B type I (SR-BI), allows CPP passage across the BBB [194].

PPMOs may effectively cross the BBB and reach targets in the CNS if administered via intrathecal injection; however such an invasive strategy, apart from causing discomfort to the patient, can also have multiple side-effects [61,196]. Once PPMOs manage to reach the CNS, vascular barriers may act beneficially to limit their escape towards the periphery, avoiding rapid loss of the drug through peripheral metabolism [64]. However, numerous risk factors associated with intrathecal administration such as infection, spinal headache, neurological injuries, have prompted researchers to further explore intravenous administrative routes in animal models. An arginine rich CPP-conjugated PMO was efficiently delivered to cerebellum and Purkinje cells when administered via tail vein in mice [197] raising hopes that systemic administration of PPMO could become a convenient route to effectively target CNS in the future. Systemic delivery of tricyclic DNA, a conformational constrained oligonucleotide analog, resulted to dystrophin restoration in the brain of *mdx* mice, although the cellular internalization mechanism of tricyclic DNA and thus the mode of endothelial barrier crossing has not been clarified by the authors [112].

SMA is an autosomal recessive disease caused by progressive loss of spinal motor neuron which leads to muscle atrophy, motor impairment and in its severe Type I manifestation, premature death [198]. It is caused by homozygous deletion or mutations in the survival motor neuron gene (*SMN1*) whereas phenotypic variations can be attributed to the number of copies of *SMN1*'s centromeric homolog, called survival motor neuron gene 2 (*SMN2*) [199]. Most *SMN2* transcripts lack exon 7 and interferes with SMN's ability to oligomerize, so the resulting protein product is rapidly degraded [200,201]. Therefore *SMN2* may only partially compensate for the lack of *SMN1* [202]. Inclusion of *SMN2* exon 7 by targeting its splicing regulatory elements that has successfully increased full-length SMN2 production *in vitro* [203,204] and *in vivo* [205], has now become the most prominent ASO therapy-based approach for SMA [206]. ASOs of 2′-OMe chemistry administered by intracerebroventricular bolus injection was successfully taken up by neurons and glial cells in the CNS in SMA model mouse, inducing exon 7 inclusion and improving function and survival of diseased mice [205,207]. Nusinersen (ISIS 396443, Spinranza®), a modified 2'-MOE PS ASO, originally developed by marketed by Biogen is a recently approved FDA drug for the treatment of SMA. Nusinersen's intrathecal administration resulted in significant improvement of motor milestones and prolonged lifespan of SMA patients with minimal adverse effects [208,209]. To improve systemic delivery and minimize side effects that result from repeated intrathecal injections, PPMO trials have been conducted. Systemic intravenous delivery of Pip6a PMO increased brain and spinal cord *SMN2* expression and rescued disease phenotype in the Taiwanese severe SMA mouse model [210]. Recent research has demonstrated that a derivative of an ApoE (141-150) peptide was capable of successfully inducing pre-mRNA exon 7 inclusion of *SMN2* in a mouse model of spinal muscular atrophy, ameliorating the phenotype of diseased mice [196].

While in SMA, like DMD, PPMO treatment focuses on increasing the production of functional proteins, in all other neurodegenerative PPMO clinical trials the main scope is to reduce aberrant mRNA transcripts [211]. In HD, expansion of the CAG sequence inside *Huntingtin* gene results in an elongated glutamine stretch near its amino terminus. The protein product is toxic and leads to neuronal loss mainly in the striatum and cortex in affected individuals [212] that suffer from progressive cognitive and motor impairment and succumb 15–20 years after the clinical onset. ASO technology selectively silenced mouse *Huntingtin* at identified exonic and intronic single nucleotide polymorphism sites in *vitro* and *in vivo* [213]. PMOs designed to target CAG repeat expansions and administered via intracerebroventricular injection significantly decreased Huntingtin's protein expression and reduced neurotoxicity in a transgenic HD mouse model [214]. Modulation of the epigenetic regulator called repressor element-1 silencing transcription factor through ASO exon skipping in a striatal cell model of HD rescued transcription of neuronal genes, proving that exon skipping may prove beneficial in HD clinical trials in the future [215]. A 2′-MOE chemistry-based drug (IONIS-HTTRx) delivered via intrathecal injection in early stage HD patients achieved a dose-dependent reduction in mutant huntingtin with no adverse effect [216].

In ALS, degeneration of upper and lower motor neurons leads to progressive and irreversible paralysis and ultimately death of the affected individuals [217]. Mutations in the *Cu/Zn superoxide dismutase 1 (SOD1)* gene have been linked to both sporadic and familial ALS [218,219], thus this gene is one of the main targets in ALS clinical trials [220]. Intrathecal delivery of a 2′-MOE ASO (ISIS 333611) decreased *SOD1* mRNA in spinal cord of recruited ALS patients in a phase 1 randomized control trial and demonstrated excellent safety profile [221] albeit concerns regarding toxicity remain [61]. Another 2′-MOE ASO compound targeting *SOD1* (IONIS-SOD1Rx, BIIB067) was investigated in a placebo-controlled phase I/II trial [222] in order to establish dosage and address safety issues and is currently in phase III clinical trial (NCT02623699). Use of morpholino oligomers to silence *SOD1* after disease onset reduced microgliosis and increased motor neuron survival in a mouse model of ALS [223]. Furthermore, targeted degradation of the hexameric expansion containing RNA foci in the *C9orf72* gene using ASOs reduced accumulation of expanded foci and dipeptide repeat proteins without affecting the overall level of *C9orf72*-encoding mRNAs in patient cells [224].

It is evident that since PMO therapeutic based strategy is already successfully applicable to many neurodegenerative diseases, the most potent PPMOs may hold tremendous therapeutic potential in this field as well as other ASO chemistries.

7. Limitations in PPMO use and future challenges

Targeted exon skipping by PPMOs is a revolutionary treatment that in the future could become applicable to a majority of DMD patients. Used alone or in combination with already approved treatment regimes, exon skipping may aid restoration of a partially functional dystrophin in most human tissues, significantly impacting on the quality and duration of life of dystrophic patients [173]. Recently, Sarepta Therapeutics announced initiation of a Phase I/IIa clinical trial of the novel PPMO SRP-5051, targeting DMD patients amenable to exon 51 skipping (NCT03375255). Pre-clinical studies using five more PPMO drug candidates targeting exons 44, 45, 50, 52 and 53 of *DMD* are also part of Sarepta's pipeline now.

Currently, the main limitation in conducting future clinical trials for PPMO based drugs is their toxicity [125,130,162]. While not well

understood, toxicity may be dependent on the following factors: species, duration of treatment, a frequency of systemic administration, dosage, exon chosen to be skipped, the peptide's cationic nature [147]. The toxicity might arise due to immunogenic mechanisms such as complement activation [167,225] and first-generation arginine-rich peptides were found to be more immunogenic than PMOs [164]. Furthermore cell-mediated and humoral responses due to repetitive PPMO treatment or serum circulating antibodies directed against the newly synthesized dystrophin are listed as potential causes of toxicity [226]. Mild manifestations of drug toxicity after low dose systemic administration in rats include lethargy and weight loss [135], but at higher doses, elevated creatinine and BUN were recorded. Similarly, AVI-5038 a PPMO targeting the human dystrophin exon 50, was found to be well tolerated at low doses, however ongoing prolonged intravenous administration caused proximal tubular degeneration in the kidneys of cynomolgus monkeys [227]. Systemic administration of PMO in monkeys has caused tubular injury, with basophilic granulation and tubular vacuolation in the examined kidneys which were deemed to be dose-dependent and reversible upon discontinuation of treatment [228]. A pre-clinical trial using an arginine rich PPMO conducted by Sarepta had to be terminated due to the toxic side effects, that could partly be attributed to the high dosage used [147]. Novel biomarkers of acute kidney injury such as Kim1 and neutrophil gelatinase-associated lipocalinare (N-GAL) are highly specific, sensitive and inexpensive and greatly facilitated monitoring the efficacy of experimental treatments in animal models [229]. In fact, the latest generation of Pip peptides (series 7,8 and 9) purposely designed to contain a reduced number of arginine residues (from 10 to 6) compared to the Pip6 series have shown dramatically improved toxicity profiles whilst maintaining the compound's splicing potency, rendering their therapeutic index more favourable for clinical development [230]. Further work in a novel PPMO series carried out in our group in collaboration with a new spin out company called Pepgen has resulted in a novel series of PPMO compounds called DPEP with favourable toxicity profiles well suited for future clinical trials. However, it still remains a challenge to estimate any side effect of long-term administration of PPMO in humans prior to we fully understand the pathophysiology of their toxicity.

Dosage and treatment regime is also of pivotal importance in obtaining maximal dystrophin expression while impeding development of severe off-target effects. The off-target effects arise from hybridization-mediated mechanisms and were not an issue for the first generation ASOs due to their limited ability to penetrate cellular membranes [63,231]. Unfortunately, while PPMO conjugation has maximized target delivery [135] it has also facilitated penetration in organs such as the liver, raising concerns about toxicity side effects [232]. Using lower dose or spacing systemic injections at longer intervals during PPMO administration might minimize side effects and potentially make the treatment cost-effective and friendlier to patients. For example, careful selection of a PMO dosing regime in mice allowed significant improvement of DGC expression complex, minimizing the histopathological features of DMD [233]. However the same group showed that intermittent injections with PMO irrespectively of the dosage could not prevent degeneration/regeneration cycles between treatments leading to muscle damage and uneven distribution of dystrophin among tissues, as occurred in the case of the eteplirsen clinical trials [117]. Therefore, prior applying any PPMO treatment regime to DMD patients, the *in vivo* efficacy of PPMO to prevent degeneration-regeneration must be carefully calculated.

The timing of exon skipping treatment is equally important to the dosage regime. If treatment is applied at the onset of disease or prior manifestation of a more severe phenotype, chances to slow down its progression are maximized, as already demonstrated in the *mdx* mouse models [157,158,173]. Indeed, delayed onset of treatment in the eteplirsen clinical trials failed to prevent loss of ambulation [91]. It is now established that destabilization of the interactions of satellite cells with the surrounding environment leads to a exacerbation of inflammation and fibrosis, further complicating DMD pathogenesis [234]. Understandably, even if a successful exon skipping restores dystrophin expression in several muscle groups in advanced diseased patients, the newly produced dystrophin won't be able to reverse those pathological processes that have already taken place due to the destabilization of the DGC complex. More importantly, the beneficial effect of any genetic correction of the DMD defect may be obscured or hampered by the quality of muscle such as advanced fibrosis, exhaustion of the satellite cell pool and reduced myofiber production that are common pathological changes observed in dystrophic muscle. It is therefore not surprising that PPMO treatment is not effective in advanced disease patients where a prolonged absence of dystrophin has led to advanced fibro-fatty degeneration, conditions which do not create an ideal environment for dystrophin restoration. This vicious cycle can only be broken if combination of existing therapies with PPMO skipping are applied, to improve the quality of life for patients.

Systemic delivery of ASOs has been majorly improved with peptide conjugation however tissues like heart and diaphragm do not demonstrate high efficient exon skipping and sustained expression of dystrophin remains challenging. Although most studies attribute this to the impermeable nature of cardiomyocytes, the cardiac levels of a PPMO injected in *mdx* mice as measured by ELISA were comparable to those of the other tissues [232], hinting that the poor efficacy of PPMOs in the heart may not necessarily be connected to their poor delivery but to their mode of subcellular uptake. A major drawback in order to optimize delivery in tissues is the lack of prediction of PPMO efficiency from their secondary structure [63]. The ability of PMO to successfully induce exon skipping of the *DMD* gene transcript depends on many factors such as their length, their affinity of binding, proximity to the acceptor splice site, ability to block an exon splicing enhancer or interference with serine/arginine protein binding [235]. *In silico* pre-screening models based on measurement of parameters such as the binding energetics of ASO to the RNA, the distance of the target site from a splice acceptor site may give up to 89% accurate prediction of the PMO's exon-skipping efficacy [236].

The animal PPMO studies undertaken so far have been very promising however major care should be taken prior to translating these findings in human studies. In fact, lack of understanding of optimization of PPMO exon skipping efficiency *via* structural modification hinders the path for discovery of more potent PPMO cocktails. Current exon skipping therapy can target only one exon and thus is applicable to a limited number of individuals harbouring the specific mutation. Ongoing clinical trials with PMOs targeting exon 53, 45 and 52 are conducted by Sarepta Pharmaceutics. Even so, none of the mentioned regimes can address patients that harbour mutations in other exons. Exons 3-9 [237] and 45-55 [238] are mutational hotspots in the *DMD* gene accounting for 7% - 47% of patients. Therefore, if a multi-exon skipping treatment regime covering these two hot spot regions will be approved, this treatment may be applicable to almost half of the DMD community. At the same time, a drug that targets multi-exon skipping of exons 45–55 would render patients asymptomatic, circumventing the unknown truncated protein stability/function factor issue, as this type of DMD patient exhibit only mild characteristics of disease [239]. Detection of truncated DMD mRNAs around the mutational hot spot reveals the presence, among others, of lowly expressed exon 44-56 multi-exon skipping product of DMD mRNAs which is an ideal induction target for exon skipping therapy [240]. Studies in myotubes transdifferentiated from DMD patient fibroblast cells recently published demonstrated that PMO oligomers could produce a dose-dependent exon 45-55 skipping, indicating that such a therapeutic effect could be more effectively achieved using PPMOs in the near future [239].

The future goal should be a development of a genetic strategy that can apply to all individuals with DMD [241]. In order to achieve an efficient multiple exon skipping that could be potentially applicable to 90% of patients [242] we need to administer PPMOs as a cocktail rather than single drugs. This will require synthesis and screening of different

oligomers as well as validation of their safety and efficacy throughout clinical trials. Such an approach will inevitably increase the cost of treatment as well as the risk of potential immunological complications arising by a combination of different chemical substances.

Finally we have to keep in mind that a rather conservative approach is required when reviewing and validating drug efficacy data that are solidly based on dystrophin quantification; because a clear, dose-response effect on the DMD phenotype positively correlating to the amount of dystrophin protein quantified from muscle biopsies has not been established yet [243]. *In vitro* exon skipping efficacy and quantification of dystrophin expression is mainly performed using primary cell models but such lines are hard to maintain and alternative screening systems are currently under investigation [244]. A more objective post-therapeutic evaluation of muscle function recovery is pending due to the inadequacy in assessment of muscle function in the clinical situation. Besides, muscle biopsy is a highly invasive technique, particularly difficult to excuse its necessity or intensify its frequency if the patients involved are children. Physical evaluation of a patient's motor activity is of pivotal importance, however this can only be done when the patient visits the hospital and fails to monitor progress in daily tasks [99] and data obtained from functional measures such as the 6MWT were very debatable at all previous clinical trials, leading to the exclusion of many patients [91]. Identification of novel pharmacological biomarkers will hopefully allow for a more patient-friendly assessment approach of PPMO therapeutic effect. Such an example of biomarkers are miRNAs that are released into the bloodstream of DMD patients as a result of fiber damage, can be detected in a serum sample and their fold change elevation levels coincide with the severity of disease whilst their reduction is associated with positive response to exon skipping treatment and dystrophin restoration [245,246].

So what steps we need to take in order to facilitate translation of these novel PPMO treatments to the clinic? It is clear that a multidisciplinary approach is needed to ensure safety of future clinical trials. Firstly, it has to be noted that although a great number of DMD animal models is currently available none of these models can perfectly assimilate human phenotype, posing significant hurdles in drug testing. The widespread used *mdx* murine model exhibits the pathological characteristics of disease but has a mild phenotype and normal life span and switching to larger mammals where we can more accurately evaluate key clinical milestones poses ethical and financial issues that are prohibiting factors in maintaining such colonies in many research facilities [247]. Therefore it is a priority to establish more appropriate preclinical models of disease. Human induced pluripotent stem cells (iPS) have been successfully programmed to produce skeletal muscle constructs expressing the characteristic markers of maturation such as MyoD and myosin heavy chain. Such iPS cells deriving from dystrophic patients can be used in order to create 3D skeletal muscle platforms that are accurately portraying the cellular hallmarks of disease [248]. Multiple studies have been conducted using patient derives iPS cells in order to evaluate approaches of dystrophin restoration such as exon skipping, exon knock in, CRISPR-Cas9 and thus development and evaluation of novel drugs [249–251]. In the future successful engraftment of corrected patient-derived iPS cells may turn to be a safe *ex vivo* applied gene therapy.

Variability in age and severity of clinical symptoms amongst the same species are common factors not only in existing DMD animal models but also in human patients. For that reason, there is a need to increase the number of clinical trials in humans in order to establish proof of concept with emphasis should be given to small clinical trials. Adequate preparation of necessary start up registries and follow up documents containing a detailed natural history of each patient is pivotal in order to accurately monitor beneficial and potential side effects of the drugs tested. Since the onset and clinical course of DMD is well described, emphasis by clinicians is currently given towards accurately monitoring disease milestones and progress (ability to walk, stand, climb stairs); therefore 6MWT test and timed function tests represent the current clinical endpoints [252,253]. Biochemical endpoints are correlated with the ability of gene therapy treatments to restore dystrophin and reduce fibrosis and to correlate such dystrophin production with improved muscle strength and physical activity especially in younger patients. The closest monitoring and pairing of both biochemical and clinical end points is essential in order to evaluate drug efficacy in early clinical trials. Improvement of existing clinical endpoints can be achieved by the use of novel technology; e.g. more sophisticated devices to effectively monitor and evaluate physical activity in patient's natural environment.

8. Conclusions

Novel therapeutic strategies using antisense oligonucleotides have tremendously altered the clinical outcome, life expectancy and prognosis in patients in the field of neuromuscular disease. Over the last decade, two ASO based drugs have obtained approval from the FDA for the treatment of DMD and SMA respectively, paving the way for novel discoveries in molecular therapy. PPMO compounds show increased efficacy as splice correcting agents at lower doses than naked ASOs and effective restoration of dystrophin body wide distribution in skeletal tissues when administered systemically. Unfortunately attempts to utilize PPMOs in the treatment of neurodegenerative diseases have so far been plagued by their lack of delivery to the CNS plus their toxicity. Ongoing research will aid clarification of the pharmacodynamics and mode of action of PPMOs and will unravel their mechanism of beneficial action. This will hopefully enable synthesis of novel PPMO drugs that will minimize off-target effects and maximize efficient uptake in skeletal, respiratory and cardiac tissues and ultimately in the CNS, bringing hope for a better quality of life to patients.

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