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1 **Current status of antisense oligonucleotide-based therapy in neuromuscular disorders**

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17 **Short title:** Antisense oligonucleotide-based therapies
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19 **Key points:**

20 ASO hold great promise as a therapeutic platform for the treatment of neuromuscular disorders.

21 The clinical benefit of ASO-therapy still highly depends on the target tissue and route of
22 administration.

23 Development of more potent chemistries or novel delivery systems is still required to improve
24 targeted delivery to muscles.
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ABSTRACT

Neuromuscular disorders include a wide-range of diseases affecting the peripheral nervous system, which are primarily characterized by progressive muscle weakness and wasting. While there were no effective therapies until recently, several therapeutic approaches have advanced to clinical trials in the past few years. Among these, the antisense technology aiming at modifying RNA processing and function has remarkably progressed and a few antisense oligonucleotides (ASO) have now been approved. Despite these recent clinical successes, several ASO have also failed and clinical programs have been suspended, in most cases when the route of administration was systemic, highlighting the existing challenges notably with respect to effective ASO delivery.

In this review we will summarize the recent advances and current status of antisense based-therapies for neuromuscular disorders, using successful as well as unsuccessful examples to highlight the variability of outcomes depending on the target tissue and route of administration. We will describe the different ASO-mediated therapeutic approaches, including splice switching applications, steric blocking strategies and targeted gene knock-down mediated by ribonuclease H recruitment. Throughout this overview, we will discuss the merits and challenges of the current ASO technology, and discuss the future of ASO development.

1 **1. INTRODUCTION**

2 Antisense oligonucleotide (ASO)-based therapeutics have made tremendous progress in the last 20
3 years and the recent approval of several drugs have increased the interest in the field even more.

4 ASOs are typically synthetic single stranded oligonucleotides of 12 to 30 nucleotides long¹⁻³, designed
5 to bind to a messenger RNA (mRNA) or non-coding RNA through Watson-Crick base pairing in order
6 to modulate their function/expression. ASOs were first used in 1978 by Zamecnik and Stephenson as
7 a gene silencing approach⁴, but the technology has rapidly evolved since then with numerous other
8 possible applications making them a valuable tool for genetic-based therapeutics⁵. While the first
9 unmodified ASOs composed of a phosphodiester backbone and sugar rings were rapidly degraded
10 within cells and bloodstream⁶, various modifications have progressively been introduced in their
11 chemical structure in order to protect them from nuclease activity and increase their stability and
12 affinity to target RNA⁷. One of the first modifications introduced was the replacement of one of non-
13 bridging oxygen atoms in the phosphate group with a sulfur atom resulting in phosphorothioate (PS)
14 ASO. This substitution increases the resistance to nuclease degradation and still supports
15 ribonuclease H (RNase H) activity to degrade the target mRNA or mutant toxic mRNA. In addition to
16 their endogenous nuclease resistance, PS modifications offer a significant advantage in terms of
17 pharmacokinetics. Their enhanced affinity for numerous proteins, including plasma, cell surface and
18 intracellular proteins^{8,9} facilitates distribution and cellular uptake of PS-ASOs compared to their
19 phosphodiester (PO) counterparts. However, PS modifications are also known to cause undesirable
20 effects due to plasma protein binding¹⁰, raising questions for their safety in humans¹¹. Acute
21 reactions of PS backbones may include immune-cell activation¹², complement activation¹³ or
22 prolongation of coagulation times^{14,15}; known to be transient and normalize when ASOs are cleared
23 from the bloodstream. Despite these safety concerns, PS backbones remain one of the most largely
24 used chemical modifications to protect ASOs from nuclease activity and increase their stability¹⁶.

25 To further improve nuclease resistance and RNA binding affinity, second generation ASOs were
26 developed with introduction of a chemical modification in the 2'-position of the sugar moiety such as

1 2'-O-methyl (2'OMe), 2'-O-methoxyethyl (2'MOE), 2'-fluorinated (2'F) and 2'-O-aminopropyl analogs¹⁷.
2 Interestingly 2'-modified ASOs were reported to reduce immune stimulation side effects compared
3 to PS-modified ASOs¹⁸. Chemical ASO modifications have kept evolving with substantial changes in
4 the sugar and have led to a wide variety of designs such as morpholinos¹⁹, locked nucleic acids
5 (LNAs)²⁰, 2'-4'-constrained ethyl (cEt)²¹ or peptide nucleic acids (PNAs)²².
6
7 ASOs can interfere and modulate RNA function via multiple mechanisms which will impact their
8 design and chemical modifications. Several reviews have previously detailed the various molecular
9 mechanisms through which an ASO can impact gene expression²³⁻²⁵. Briefly, ASOs can be used to
10 promote RNA degradation via the recruitment of RNase H1 which will cleave the mRNA-ASO hetero-
11 duplex while leaving the ASO intact (figure 1, left panel). It is important to note that most sugar
12 modified ASOs became unable to elicit RNase H1-mediated RNA degradation, hence ASOs must be
13 designed as "gapmers" with a central core of 8 to 10 consecutive DNA nucleotides to support binding
14 and cleavage by RNase H1. Alternatively, ASOs can be fully modified and work through steric
15 hindrance mechanisms either to modulate splicing (in which case they may be called splice switching
16 oligomer - SSO), inhibit RNA toxicity through the disruption of RNA structure, impact polyadenylation
17 selection site, inhibit translation, or bind to microRNAs to block their interaction with mRNA (figure
18 1, right panel). The variety of mechanisms through which ASO can act and the subsequent
19 therapeutic applications truly represent unique opportunities for the treatment of many conditions.
20 Steady progress in chemistry and design over the years have led to several approved ASO and
21 numerous others currently being tested in clinical trials, in particular for neuromuscular diseases.
22 These recent successes elicit optimism and hope for the development of future therapeutics using
23 the antisense technology. However, the clinical benefit of ASO therapy still very much depends on
24 the target tissue and route of administration. The therapeutic potential of systemically delivered
25 ASOs remains far behind since it is assumed that only about 1% of ASO reach the correct cellular
26 compartment after intravenous or subcutaneous administration²⁶. In this review, we will discuss the

1 recent advances and current status of ASO therapies for several neuromuscular disorders including
2 Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA), myotonic dystrophy (DM1),
3 amyotrophic lateral sclerosis (ALS) and Huntington disease (HD). Through these examples, we will
4 cover the broad range of neuromuscular disorders from the muscle to the more neurodegenerative
5 end of the spectrum, like ALS and HD, to reflect the variation in clinical benefit depending on the
6 target tissue/route of administration, but also with respect to the diversity of chemistries and
7 mechanisms of action.

8

9 **2. Duchenne muscular dystrophy**

10 With an incidence of one per 5,000 boys on average²⁷, Duchenne muscular dystrophy is the most
11 common muscular dystrophy. DMD is caused by mutations, typically large deletions (approximately
12 65%) in the DMD gene, disrupting the open reading frame and leading to non-functional or absent
13 dystrophin protein. As dystrophin plays a major mechanical and signalling link between the actin
14 cytoskeleton and the extracellular matrix, its absence leads to progressive muscle wasting and
15 premature death of patients. In contrast to the frame shifting mutations observed in DMD, mutations
16 maintaining an open reading frame allow the production of a partially deleted but, in most cases,
17 functional dystrophin. This is observed in the milder phenotype, Becker muscular dystrophy (BMD),
18 which results in a later age of onset and milder clinical involvement (ranging from intermediate to
19 asymptomatic). Moreover, about 50% of DMD patients have individual dystrophin-positive muscle
20 fibers²⁸, called revertant fibers, with protein correctly localized at the membrane. It has been shown
21 that they come from a spontaneous skipping of several exons allowing to restore an in-frame
22 transcript²⁹. Altogether these observations led to the emergence of a novel therapeutic approach
23 named exon-skipping using ASO to modulate the pre-mRNA splicing (figure 1, right panel). The
24 principle to restore an opening reading frame by exon-skipping therapy for DMD has first been
25 demonstrated more than twenty years ago in culture cells²⁹. Since, numerous *in vivo* studies
26 performed in several animal models have provided pre-clinical evidence for the therapeutic potential

1 of antisense strategy. Although the exon-skipping approach appears to be able to restore the reading
2 frame in a large proportion of patients (possibly up to 83% of all DMD patients³²), this strategy will
3 not offer a definite cure but an improvement towards a BMD-like phenotype depending on the
4 functionality of the restored dystrophin, as not all protein domains are dispensable. Favorably, the
5 majority of deletions clusters into hotspot regions between exons 43 and 53, suggesting that skipping
6 of the same group of exons is applicable to large groups of patients. Notably, exon 51 skipping
7 (applicable to 13% of all patients) was the primary choice for numerous phase 1 clinical trials, which
8 evaluated various chemistries of antisense oligonucleotides as detailed below.

9

10 **2.1. 2'-O-Methyl-PS RNA.**

11 While most of the preclinical work using 2'OMe-PS ASO for DMD was produced by the LUMC (Leiden
12 University Medical Center)-based research groups, first clinical data were actually published by a
13 Japanese group in 2006 using a PS DNA ASO (Table 1). A single patient, with an exon 20 deletion, was
14 injected with a 31-mer ASO targeting the exon 19 of the DMD gene (named AO19) in the peripheral
15 vein at 0.5 mg/kg four times at one-week intervals³³. The treatment was well tolerated and resulted
16 in the detection of skipped transcript in lymphocytes (despite this target being questionable), and
17 traces in biopsied biceps muscle. Nevertheless, the novel transcript did not allow the restoration of
18 quantifiable dystrophin expression and no follow-up studies were ever conducted.

19 In parallel in Europe, a 20-mer 2'OMe-PS ASO targeting exon 51 was developed and shown to restore
20 significant levels of dystrophin in mouse models of DMD³⁴. Based on these encouraging preclinical
21 data, the Dutch group in collaboration with Prosensa evaluated the intramuscular injection of this
22 ASO named drisapersen in four DMD patients eligible for exon 51 skipping³⁵. The treatment was well
23 tolerated and confirmed the proof of principle of the ASO-mediated exon skipping approach for DMD
24 since some dystrophin expression was detected, supporting the development of further clinical
25 investigations. Results of the phase 1/2a trial evaluating the systemic administration of drisapersen
26 were published in 2011³⁶. Four groups of 3 DMD boys received abdominal subcutaneous injection at

1 doses of 0.5, 2, 4 and 6 mg/kg/week of 2'OMe-PS ASO for 5 weeks followed by a 6 to 15 months off-
2 treatment and a 12-week extension phase at 6 mg/kg/week. While no serious adverse effects were
3 reported, a total of 120 mild or moderate adverse events occurred, notably proteinuria and several
4 types of reactions at the injection site. Tibialis anterior muscle biopsy samples were analyzed at 2
5 weeks and 7 weeks after the end of the dose-escalation phase. Exon 51 skipping was observed in all
6 patients receiving 2, 4 or 6 mg/kg/week at both time points but with variable extent. Quantification
7 of western blot shows that dystrophin signal intensity was 1.5 to 8.2 times greater than the baseline
8 condition (taken from 0.5 mg/kg group of patients) in the groups receiving 4 and 6 mg/kg/week, 2
9 weeks after the end of the treatment. Following 12 weeks of treatment, no increase in specific
10 muscle force was observed but there was average improvement of 35.2 ± 28.7 m in the distance
11 walked in 6 minutes (6MWD). Those 12 patients entered a long-term 188-week extension study with
12 subcutaneous injection of 6 mg/kg/week initially³⁷. Similar adverse effects were reported, including
13 injection-site reactions (erythema, hematoma, and induration), increased urinary α 1-microglobulin,
14 and proteinuria. Biopsies were taken from the tibialis anterior muscle at week 24 for all subjects and
15 at either week 68 or 72 for volunteering subjects. Although exon-skipping and dystrophin were
16 detected at both time-points, no formal conclusion could be drawn on potential increase from
17 baseline because no material from pre-treatment biopsies was available. At the end of the
18 treatment, 2 patients were non-ambulant and 2 were unable to complete the 6MWD at later visits.
19 Nevertheless, on the 8 remaining subjects, the 6MWD appeared improved by a median increase of
20 64 meters (mean=33) and the cardiac function showed stability but on the other hand, muscle
21 strength decreased during the study. Interestingly though, despite the heterogeneity of subjects,
22 some of them who were in a decline stage of the disease maintained ambulation throughout the
23 study. Based on these relatively encouraging data, an open-label extension phase 3 study was
24 launched with 186 ambulant subjects³⁸. Unfortunately, the slight difference observed in the 6MWD
25 from baseline was found non-significant, which was thought to be largely due to subgroup
26 heterogeneity. A post-hoc analysis, performed on a subgroup of 80 subjects with a baseline 6MWD

1 between 300 and 400 meters, revealed a treatment benefit in 6MWD of 35.4 meters (95% CI: 1.8,
2 69.0), supporting early intervention. The inability to meet the primary efficacy endpoint
3 (improvement in 6MWD) together with the significant adverse effects (injection-site reactions and
4 renal issues) led to the rejection of drisapersen's application by the US Food and drugs
5 Administration (FDA). By mid-2016, Biomarin announced the withdrawal of drisapersen market
6 authorization application in Europe and the discontinuation of all their ASO programs for DMD which
7 included exons 44, 45 and 53 to invest in research of next generation ASOs.

8

9 **2.2. Phosphorodiamidate Morpholino Oligomer (PMO)**

10 In parallel to the 2'OMe-PS ASO development, another chemistry of ASO was developed for DMD:
11 Phosphorodiamidate Morpholino Oligomers also called PMO. Following numerous preclinical studies
12 showing encouraging results³⁹, the UK MDEX consortium conducted a phase 1/2 trial with a 30-mer
13 PMO targeting exon 51, named eteplirsen, in seven patients. Intramuscular injection of eteplirsen
14 was well tolerated by all patients and intensity of dystrophin staining increased to 42% of normal
15 levels in dystrophin positive fibers of patients treated with the higher dose⁴⁰. While intramuscular
16 injection was a valuable proof-of-concept, the main challenge in DMD is to target cardiac and
17 respiratory muscles therefore systemic delivery was next assessed. Sarepta started a dose escalation
18 study from 0.5 to 20 mg/kg with multiple injections⁴¹. Adverse events were generally mild to
19 moderate and a dose-dependent significant increase in dystrophin expression was observed and
20 associated with α -sarcoglycan and neuronal nitric oxide (nNOS) restoration at the sarcolemma.
21 Following these encouraging results, phase 2 studies with higher doses (30 and 50 mg/kg) were
22 launched⁴². Eteplirsen injections resulted in increased dystrophin production in all patients following
23 at least 24 weeks of treatment with a statistically significant difference in the 6MWD in some
24 patients, depending on the age-related disease progression⁴³. Based on detection of dystrophin
25 protein (although minor) and the absence of adverse events, eteplirsen was approved by the FDA in
26 2016, under the accelerated approval pathway, despite no measures of muscle function. Although its

1 approval was shrouded in controversy, eteplirsen became the first approved drug for DMD in the US,
2 as well as the first approved splice-switching ASO⁴⁴. Sarepta was asked to perform confirmatory
3 studies to establish a clinical benefit which had not been demonstrated. In 2019, they started to
4 recruit patients for a phase 3 study with intravenous (IV) injections of two different high doses (>30
5 mg/kg). In the meantime, they reported functional data from two non-ambulatory patients
6 compared to 10 patients who remained ambulatory⁴⁵ and showed that despite the loss of
7 ambulation, their cardiac, pulmonary and upper limb functions remained relatively stable and the
8 increase in dystrophin production (less than 2%) was similar to those of ambulatory patients. As
9 eteplirsen was well tolerated over 3.2 years, Sarepta performed a phase 3 study with 7 to 16 years
10 old patients and a phase 2 study on younger child (4-6 years old), but no results have been published
11 yet.

12 Regardless of eteplirsen's approval by the FDA in 2016, two years later the European Medicines
13 Agency (EMA) gave a negative opinion for this treatment⁴⁴. In their public report, the Committee for
14 Medicinal Products for Human Use (CHMP) considered that the safety profile of eteplirsen had not
15 been thoroughly characterized and they mentioned that a sufficient number of patients compared to
16 placebo would be needed to conclude. They concluded that the current benefit-risk balance was not
17 positive since the efficacy was not sufficiently demonstrated. This difference between the two
18 agencies highlights their dissimilar views on the role of dystrophin as a surrogate endpoint. Despite
19 this setback in Europe, Sarepta continued the clinical development of their PMO-ASOs notably
20 targeting exons 45 and 53. Toxicology results in non-human primates suggested an encouraging
21 safety profile of these two PMOs, SRP-4045 (Casimersen) and SRP-4053 (Golodirsen), similar to
22 eteplirsen⁴⁶. Findings were mostly limited to the kidneys, which presented minor microscopic
23 changes that were non-adverse after 12 weeks of infusion with SRP-4045 and SRP-4053, or 39 weeks
24 of infusion with eteplirsen at various doses (5, 40 and 320 mg/kg/week)⁴⁶. Based on positive results
25 from a phase 1/2 clinical trial⁴⁷, golodirsen received in December 2019 its first global approval in the
26 USA for the treatment of DMD patients amenable to exon 53 skipping⁴⁸.

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Another company, NS-Pharma, in collaboration with Japanese groups, used another optimized sequence named Viltolarsen (NS-065/NCNP-01) to target exon 53 but with much higher doses (up to 80 mg/kg). In a phase 1 dose-escalation trial, the increase of dystrophin was negligible, except for one patient with a dystrophin/spectrin ratio increased by 8.1%, correlated to a significant skipping in the higher dose group⁴⁹. No serious adverse events were reported and the observed drug reactions were common for ASOs such as proteinuria, albuminuria or increase in N-acetyl-β-D-glucosaminidase but also anemia. Following this phase 1, parallel trials in USA/Canada and in Japan have been completed. During the Japanese phase 1/2 study with the higher doses of 40 and 80 mg/kg/week for 24 weeks, the increase of dystrophin expression was between 0.13 and 2.78% respectively. In parallel, the North American phase 2 study with the same doses showed that the average increase in dystrophin protein was about 5% from baseline⁵⁰. However, it is important to note that there are no standardized methodologies for dystrophin quantification between those studies, despite the recent emergence of alternative methods to quantify dystrophin, such as mass spectrometry, capillary western immunoassay or immune-affinity liquid chromatography⁵¹. Based on the presence of dystrophin restoration (without measurement of muscle function), and the absence of serious adverse effects Viltolarsen was approved in March 2020 and thus became the first ASO to be approved by the Japanese agency.

In spite of these recent approvals suggesting some success, the levels of restored dystrophin in biopsies from DMD patients treated with these compounds remain extremely low. It is therefore unclear at the moment whether these drugs can truly achieve clinically relevant outcomes. To improve the delivery of ASOs to targeted tissues²⁶, alternative chemistries are being developed, as well as novel generations of conjugated ASOs.

2.3. Alternative chemistries and next generations of compounds

Wave Life Sciences recently developed an investigational stereopure ASO (suvodirsen), designed for exon 51 skipping, which was shown to induce enhanced exon skipping and dystrophin protein restoration in cell cultures compared with other exon-skipping compounds such as eteplirsen or drisapersen (52% dystrophin protein restoration in cells versus 1% with other exon skipping ASOs). A phase 1 clinical trial was initiated in November 2017 to assess the safety and tolerability of suvodirsen with 5 ascending doses, from 0.5 to 10 mg/kg/week. They reported infusion-associated reactions consisting of pyrexia, headache, vomiting and tachycardia, essentially for the doses equal or higher than 5 mg/kg. As patients who received 7 or 10 mg/kg met the predefined stopping criteria, the remained patients moved directly into the open label extension at the 5 mg/kg dose level⁵². In December 2019, Wave announced that interim results showed no change from baseline in dystrophin expression with either the 3.5 or 5 mg/kg doses of suvodirsen. Both the extension trial and the phase 2/3 trial were discontinued and further development of exon 53 compounds was suspended.

Among advancing chemistries, a Japanese group developed a 2'-OMe RNA/ENA (2'-O,4'-C-ethylene-bridged nucleic acid) chimera ASO to induce dystrophin exon 45 skipping⁵⁴. During the congress Myology in 2016, the group presented results from one clinical study with a 7-year old boy. After 4 weeks IV infusion at 0.5 mg/kg/week, they showed partial exon skipping and reported an increase of the 6MWD. A phase 1/2 clinical trial is ongoing in Japan to evaluate the safety, tolerability, effectiveness, and pharmacokinetics of the drug (DS-5141). DMD patients are given subcutaneous injections of DS-5141 at different doses (0.1, 0.5, 2 and 6 mg/kg) for up to 12 weeks and then enrolled in an extension protocol of 48 weeks with doses at 2 and 6 mg/kg. Results announced in April 2018 by the company revealed no serious adverse events but efficacy data were unclear with detection of exon 45 skipping in all participants while dystrophin protein was only partially

1 identified⁵⁵. The study has been updated on the US Clinical Trials website and announced a study
2 completion date in December 2020.

3

4 Conjugation of ASO to cell penetrating peptides (CPP) has also been investigated to improve delivery
5 to the muscle and peptide conjugated-PMO (PPMO) have shown encouraging preclinical data⁵⁶.
6 Despite the toxicity issues arisen in non-human primates treated with the first PPMO developed
7 against exon 50⁵⁷, newer generations of CPP with better safety profile have since been developed.
8 Sarepta has recently completed a phase 1 trial with a PPMO targeting exon 51 and is now recruiting
9 for a phase 1/2. No results have been posted or published yet.

10

11 Development of other alternative chemistries led to the identification of tricyclo-DNA (tcDNA) as a
12 promising ASO for therapeutic splice-switching applications. TcDNA have demonstrated unique
13 pharmaceutical properties and widespread uptake in many tissues after systemic delivery; including
14 the capacity to cross the blood brain barrier (BBB) at low level after systemic delivery^{58,59}. A phase
15 1/2 clinical trial is programmed for mid-2021.

16 Another alternative to overcome the delivery and potential toxicity challenges of ASO is the use of
17 viral vectors encoding small nuclear RNAs engineered to shuttle antisense sequences into cells. This
18 type of vectorization in adeno-associated virus (AAV) vector has demonstrated promising preclinical
19 data in mouse and dog models of DMD, allowing appropriate subcellular localization with pre-mRNAs
20 and long term correction⁶⁰⁻⁶². Based on these encouraging findings, a phase 1/2 clinical trial is
21 currently being planned by Audentes therapeutics in collaboration with the Nationwide Children's
22 Hospital to develop a first product candidate, AT702 targeting exon 2. Exon duplications account for
23 11% of DMD mutations and exon 2 is the most common duplication in patients. Targeting
24 duplications aims to create a full-length dystrophin (i.e. fully functional) in contrast with internally
25 deleted dystrophin resulting from the correction of frameshift or nonsense mutations.

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3. Spinal muscular atrophy

Spinal muscular atrophy (SMA) is a group of autosomal-recessive inherited disease characterized by progressive muscle atrophy due to the degeneration of α -motor neurons of the anterior horn of spinal cord. In most cases SMA is caused by homozygous deletions of the gene *SMN1* (survival motor neuron 1) encoding the SMN protein and its clinical phenotype is heterogeneous, ranging from a severe to a mild phenotype (SMA type 1 to 4). A prevalence of approximately 1–2 per 100,000 persons and incidence around 1 in 10,000 live births have been estimated with SMA type 1 accounting for around 60% of all cases⁶³. Loss of SMN leads to a progressive proximal muscle weakness and deleterious effects on respiratory function. In humans, the severity of the disease is mostly counterbalanced by the presence of the *SMN2* gene and inversely proportional to its copy number but discrepant SMA cases support the role of other phenotype modifiers⁶⁴. *SMN2* differs from *SMN1* by one nucleotide in the coding sequence: a C-T substitution at position 6 of exon 7 which results in its exclusion during the splicing of the pre-mRNA in about 90% of the transcripts, but with cells and tissues variation. This alternative spliced product lacking exon 7 is translated into a truncated protein that is degraded shortly after while the 10% correct transcript translates for a full-length functional SMN protein.

Considering that *SMN2* gene is similar in all SMA patients and that they usually have several copies of *SMN2*, modulating its splicing has become an attractive therapeutic option. The mechanism of splicing regulation is complex and involves more than 40 regulatory protein factors. Splicing of *SMN2* exon 7 requires many different elements, including a suboptimal intron 6 branch point⁶⁵, an extended inhibitory context, a conserved tract domain, an inhibitory 3'-cluster, an intronic silencer element in intron 7 (ISS-N1) and a terminal stem-loop (TSL) structure on the 5' splice site of exon 7⁶⁶. Once the genomic organization of *SMN2* was better characterized, research groups started using the antisense platform in order to force the inclusion of exon 7 (figure 1, right panel).

1 Various classes of ASOs have been developed to modulate *SMN2* mRNA splicing, including 2'OMe-PS,
2 PMO and 2'MOE-PS. The most promising candidate identified was an 18-mer 2'MOE-PS ASO
3 targeting the ISS-N1 sequence which was reported to regulate *SMN2* exon 7 inclusion⁶⁷. Initial studies
4 using this ISS-N1 ASO in a mild SMA mouse model (type 3) demonstrated rescue of tail and ear
5 necrosis following intracerebroventricular (ICV) infusion⁶⁸. A year later, the same group
6 demonstrated increased survival of type 1 SMA mice after subcutaneous injections at postnatal day
7 P0 and P3⁶⁹. Clinical trials were initiated in 2012 with this oligonucleotide, named nusinersen
8 (originally IONIS-SMNRx) by Ionis Pharmaceuticals. The first phase 1 clinical trial was conducted in
9 patients with type 2 and 3 SMA⁷⁰ and was aimed at evaluating safety, tolerability and
10 pharmacokinetics of a single intrathecal injection at different doses (1, 3, 6 and 9 mg). In this study,
11 nusinersen was not only well tolerated but an improvement of motor function was observed with the
12 9 mg dose. Moreover, with a half-life in cerebrospinal fluid (CSF) of 4 to 6 months, nusinersen allows
13 large intervals between injections. Several clinical studies quickly followed these encouraging results.
14 First, a phase 2 evaluating multi intrathecal doses of 6 to 12 mg over 32 months of treatment was
15 launched in infants with early onset⁷¹. The treatment was well tolerated and patients in the 12 mg
16 group showed improvement in motor function and an increase in electrically excitable muscle.
17 Moreover, these patients surpassed median age of death, compared to natural history cohorts.
18 Following these favorable results, a phase 3 study was also initiated with more injections in the first
19 months. Since a greater improvement was seen in patient with a shorter disease progression during
20 treatment initiation, the crucial question of a better clinical response with a pre-symptomatic
21 treatment has been examined in a phase 2 study. Interim efficacy and safety results were recently
22 published⁷² and reported that all injected patients past the age of symptom onset were alive, without
23 permanent ventilation. They achieved motor milestones and are continuing to make progress,
24 highlighting the need to start treatment before symptomatic period.

25

1 In parallel to these trials on type 1 and 2 patients, studies were conducted on patients with later-
2 onset SMA, including a phase 3 study in children over the course of 15 months⁷³ and a phase 1b/2a
3 followed by a 3-year extension study⁷⁴. They both confirmed the improvement of motor function and
4 reported that none of the adverse effect was considered drug related therefore validating the
5 effectiveness of nusinersen. In the extension study, electrophysiological outcome measures were
6 reported stable suggesting that the benefit of treatment on distal innervation capacity may be less
7 robust than the overall benefit on motor function. The authors suggested that this may be influenced
8 by different factors such as age, severity of denervation at the time of enrollment, duration of
9 exposure, and nerve muscle group tested.

10

11 Nusinersen was approved by the FDA in late December 2016 and by the EMA in June 2017.
12 Commercialized by Biogen under the name spinraza[®], this drug probably represents the biggest
13 success of the ASO-based therapy. However, many challenges remain and need to be addressed in
14 the near future. Even if nusinersen leads to global improvement, the response varies from a patient
15 to another and improvement in type 2 patients is less significant than in those with SMA type 1 and
16 there is insufficient evidence of efficacy in SMA types 3 and 4⁷⁵. Ongoing studies show that
17 presymptomatic treatment may be an important consideration; nevertheless, in patients with active
18 disease, combination of nusinersen with other drugs, including those targeting peripheral tissues
19 may also prove useful and require additional investigations. The involvement of additional peripheral
20 organs, such as cardiac dysfunctions and distal digits necrosis has indeed been reported in the
21 pathogenesis of the disease progression, especially in the most severe cases⁷⁶. The current mode of
22 administration using intrathecal injections does not address these peripheral issues and can be a risk
23 of tissue damage or infection after multiple administrations. Several groups have successfully worked
24 on alternative, albeit still lumbar, route of injection in patients with severe scoliosis or with metal
25 implantation⁷⁷⁻⁷⁹. To date, intrathecal administration is used because most PS-ASOs are not able to
26 cross the BBB despite an effective uptake by brain tissues. Promising alternatives are currently being

1 explored at the preclinical level, with PPMO⁸⁰ or tcDNA⁸¹ chemistries which have been shown to
2 cross the BBB after systemic delivery. Besides ASOs, other promising candidates administered
3 systemically are in clinical trials including gene therapy with AAV9 (IV and intrathecal administration),
4 risdiplam, acting as a splicing modifier (oral administration), anti-myostatin antibody and
5 reldesemtiv, a muscle troponin activator (oral administration) (approaches reviewed in ⁸²). Amongst
6 these, only *SMN1* gene replacement mediated by the AAV vector AVXS-101 has been approved by
7 the FDA so far and offers the advantage of a single administration in contrast with the repeated
8 injections required for nusinersen.

9

10 **4. Myotonic dystrophy**

11 Another example of inherited neuromuscular disorder which can be targeted by ASO is Myotonic
12 dystrophy type 1 (DM1), which is the most common form of muscular dystrophy in adults with an
13 estimated incidence of 1 per 8,000 individuals worldwide⁸³. DM1 is a multisystemic disease
14 characterised by myotonia, muscle weakness, cardiac arrhythmias, insulin resistance and cognitive
15 dysfunction. DM1 is caused by an abnormal expansion of a CTG trinucleotide repeat in the 3'-
16 untranslated region of the human dystrophin myotonia protein kinase (*DMPK*) gene.

17 Transcription of the *DMPK* CTG repeats into a highly structured *DMPK* CUG^{exp} RNA leads to RNA
18 aggregates, referred to as foci in which the muscle blind-like 1 protein (MBNL1) is found sequestered.

19 Limiting the availability of MBLN1 splicing regulator results in the missplicing of several important
20 muscle-expressed genes including *CLCN1*, *INSR*, *BIN1*, *DMD*, and *SCN5A* that have been associated
21 respectively with myotonia, insulin resistance, muscle weakness, dystrophic process, and cardiac
22 conduction defects, all symptoms of DM1⁸⁴. Restoring the abnormal splicing of some of these targets
23 using ASOs has been explored and the correction of *CLCN1* transcripts was achieved using a PMO for
24 example, leading to reduced myotonia in mouse models of DM1⁸⁵. However, targeting the
25 downstream effects may not address all the symptoms of the disease given the large number of
26 misspliced mRNAs in DM1. Alternatively, therapeutic ASOs targeting directly the *DMPK* RNA have

1 been developed over the years, aiming at either degrading the CUG^{exp} transcripts using gapmer ASOs
2 recruiting RNase H (figure 1, left panel) or at releasing sequestered MBNL1 from CUG^{exp}-containing
3 RNAs by direct competition using steric blocker (fully modified, figure 1 right panel) ASOs. A PMO
4 CAG25 binding to CUG^{exp} RNA was shown to block its interaction with MBNL1, disperse the nuclear
5 foci and therefore reduce the overall burden of this toxic RNA⁸⁶. Interestingly studies using steric
6 blocker ASOs (PMO as well as 2'OMe modified) found a reduction in CUG repeat transcripts when
7 these ASO should only “block” and not degrade the transcripts, therefore suggesting degradation by
8 another pathway once released from binding with MBNL proteins. Reduction of the CUG^{exp} RNA and
9 subsequent reduction in myotonia were also induced more directly using gapmer ASO targeted
10 either to the CUG repeats⁸⁷, to the 3'UTR or to the coding region of the human skeletal actin (*HAS*)
11 gene in the *HSA*^{LR} mouse model, a transgenic mouse carrying 250 CTG repeats in the 3'UTR of the
12 HSA transgene⁸⁸. Following these encouraging results, Ionis Pharmaceuticals designed higher affinity
13 ASOs containing 2'-4'-constrained ethyl (cEt) modifications, which were described with significantly
14 enhanced *in vivo* potency compared with 2'-MOE–modified ASO⁸⁹. The cEt-modified DMPK ASO
15 demonstrated potent activity in the skeletal and cardiac muscles of normal mice, as well as
16 cynomolgus monkeys⁹⁰. Moreover the same gapmer was shown to improve body weight, muscle
17 strength and muscle histology in the DMSXL mouse model, which harbors a mutant form of the
18 human DMPK gene (carrying >1,000 CTG repeats)⁹¹.

19

20 Together with a favourable safety profile, these encouraging preclinical results led to the clinical
21 evaluation of this approach in a human clinical trial (NCT02312011). While the primary objective of
22 the study was safety and tolerability, the results of the trial also revealed insufficient concentration
23 of the therapeutic ASO in muscle tissues following systemic administration and the development of
24 this drug was therefore discontinued⁸⁴. These disappointing results confirmed that delivery remains
25 one of the main challenge of the ASO-therapy for neuromuscular disorders and even more so for
26 DM1. Since the membrane integrity of muscles from patients with DM1 was found no more

1 permeable than in healthy patients⁹², in contrast to patients with DMD for example, DM1 drugs must
2 overcome the barrier of a fully functional membrane. Therefore, more potent chemistries or
3 improved delivery systems must be developed. Ionis Pharmaceuticals has recently focused on ligand
4 conjugated-ASO and published the enhanced potency of fatty acid conjugated ASO in mouse
5 muscles⁹³. In particular, palmitic acid conjugation was shown to increase ASO peak plasma
6 concentration (Cmax) and improve delivery to interstitial space of mouse muscle. However, the same
7 group also reported that the promising palmitate conjugate was found less effective in monkeys,
8 therefore limiting its therapeutic potential in humans⁹⁴.

9
10 Among strategies to improve delivery of ASOs in DM1 muscles, CPP conjugates also offer an
11 attractive option. A recent study from Klein and colleagues demonstrated that a low-dose treatment
12 with a Pip6a-conjugated PMO directed against pathogenic CUG^{exp} repeats was sufficient to achieve
13 an effective concentration of ASOs in muscle fibers and induce an efficient and long-lasting
14 correction of myotonic dystrophy features in DM1 mice as well as in patient-derived cells⁹⁵.
15 Amelioration of the DM1 phenotype included normalization of the transcriptome and reduction in
16 the prevalence of foci-positive fibers. These encouraging results support the clinical development of
17 peptide conjugates for systemic corrective therapy in DM1 but the promise of such compounds still
18 crucially depends on how well they will be tolerated in humans.

19

20 **5. Amyotrophic lateral sclerosis**

21 Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting motor
22 neurons in the cortex and spinal cord. With a prevalence of approximately 6 cases per 100 000
23 individuals, ALS is the most common motor neuron disease in adults⁹⁶. Symptoms in ALS depend on
24 the neurons affected by the disease, most patients have onset in one limb or two upper or lower
25 limbs in an asymmetric fashion and one-third have bulbar onset. Voluntary muscle action is gradually

1 affected and patients may become completely paralyzed in the later stages of the disease. In general
2 the death of patients occurs due to a respiratory paralysis generally 3 to 5 years following diagnosis⁹⁷.
3 ALS is considered as a complex genetic disorder without discernible family history in the majority of
4 cases. However, ≥ 30 genes have been reported to confer a major risk of ALS and evidence suggests
5 roles of oligogenic inheritance and of genetic pleiotropy⁹⁸. In the populations with a family history,
6 four genes account for up to 70% of all cases, namely, *SOD1*, *C9orf72*, *TARDBP* and *FUS*⁹⁸. The
7 antisense technology is an attractive option for ALS and different ASOs are currently being developed
8 preclinically and clinically; the most promising approaches targeting these genes are reviewed below.

9 **5.1. *SOD1* mutations**

10 12–20% of familial cases of ALS are due to mutations in the *SOD1* gene. More than 100 different
11 mutations in the *SOD1* gene confer a toxic gain of function of the SOD1 protein that may impact
12 neuronal function and survival⁹⁹. Reduction of the mutant SOD1 protein concentration can slow
13 progression of SOD1-linked ALS¹⁰⁰ and ASOs have therefore been developed in this aim. During the
14 preclinical studies performed in rats and nonhuman primates models, ASOs were shown to
15 effectively target the central nervous system (CNS) after intrathecal administrations, decrease SOD1
16 concentration and increase survival¹⁰¹. Following these encouraging preclinical data, ISIS333611, a
17 2'MOE-PS gapmer ASO targeting the exon 1 of *SOD1* mRNA was further developed and became the
18 first experimental ASO delivered intrathecally into patients for the treatment of a neurodegenerative
19 disease¹⁰². This clinical study confirmed that intrathecal administration of ASOs into the CNS of
20 patients was well tolerated without dose-limiting toxic effects or safety concerns¹⁰². Moreover, SOD1
21 concentrations in brain tissue were found to correlate with SOD1 concentrations in the CSF, which
22 allowed the use of CSF SOD1 protein concentrations as a pharmacodynamic marker for ASO activity
23 in the next clinical trials. Despite being encouraging, ISIS333611 effects were modest and advances in
24 ASO technology in particular for other neuromuscular disorders like SMA identified more effective
25 delivery methods to the CNS as well as more potent ASO designs. Further research highlighted a six
26 fold more effective 2'MOE gapmer (named BIIB067) targeting a different region of the *SOD1* mRNA

1 (the 3' UTR part) and resulting in increased survival of transgenic rodents compared to the previous
2 ISI333611¹⁰³. Preliminary results from the phase 1b/2a clinical study with this ASO (NCT02623699)
3 revealed that intrathecal administration of the highest dose tested of BIIB067 reduced SOD1
4 concentrations in patients CSF without toxicity, although results must be confirmed on higher
5 number of patients. Currently, two phase 3 clinical trials are ongoing with BIIB067: one to confirm
6 the efficacy, safety, tolerability, pharmacokinetics, and pharmacodynamics of the ASO
7 (NCT02623699) and another to evaluate the long-term safety and tolerability (NCT03070119). The
8 first results of these studies are expected in 2021.

9

10 **5.2. *C9orf72***

11 40% of familial cases of ALS are due to an hexanucleotide expansion (GGGGCC) in the noncoding
12 region of the *C9orf72* gene¹⁰⁴. Several potential mechanisms have been suggested to explain the
13 neurodegeneration caused by this expansion¹⁰⁵. Amongst them, a toxic gain of function of the RNA,
14 depleting RNA-binding protein similar to the mechanisms of DM1, or a decreased production of the
15 *C9orf72* have been proposed. However, the loss of *C9orf72* protein alone is not sufficient to create
16 the disease since deletion of the *C9orf72* gene in mice did not result in detectable
17 neurodegeneration¹⁰⁶. Alternatively, it was reported that the *C9orf72* mutations lead to the
18 transcription of RNAs containing sense GGGGCC (G4C2) and antisense CCCCGG (C4G2) repeats which
19 can exert toxic gain of function and drive neurodegeneration through the production of different
20 poly-dipeptides¹⁰⁷. These repeats induce an increase in 5 species of dipeptide repeat proteins:
21 poly(GP), poly(GA), poly(PR), poly(GR), and poly(PA), each shown to aggregate in CNS tissues of
22 individuals with ALS¹⁰⁸. The most important one being the poly(GP) because it is *C9orf72* specific and
23 its levels are consistent over time, supporting its continued use as a pharmacodynamics biomarker
24 for expansion-targeted therapeutics¹⁰⁸. Several studies have reported the use of RNase H1-recruiting
25 ASO and shown reduction of RNA foci and dipeptides in cellular as well as mouse models of *C9orf72*
26 ALS^{105,109}. In particular, Jiang and colleagues demonstrated that an ASO targeting the region between

1 exon 1a and 1b leads to a 60-80% decrease of repeat-containing *C9orf72* RNA levels (with exon 1a) in
2 the cortex and spinal cord and preserves *C9orf72* protein encoding RNAs (with exon 1b). Four weeks
3 after a single injection of this ASO, a 55-60% decrease of sense foci number was observed and both
4 poly(GP) and poly(GA) were decreased to almost undetectable levels¹⁰⁹. These results suggest that
5 ASO therapy can mitigate the toxicity from repeat RNAs without exacerbating the disease with a
6 potential loss of *C9orf72* function. Based on these findings, clinical studies have been initiated to
7 evaluate the safety, tolerability and pharmacokinetics of BIIB078 (an ASO targeting the same region
8 of *C9orf72* gene)¹¹⁰ administered intrathecally to ALS patients with *C9orf72* hexanucleotide
9 expansion (NCT03626012 and NCT04288856).

10

11 **5.3. Other targets for ALS**

12 The field has rapidly advanced over the past 5 years and other targets related to TDP-43, ATXN2, FUS
13 or stress pathways are also being investigated as potential therapeutics for ALS¹¹¹. FUS protein is
14 involved in RNA metabolism, including transcription, alternative splicing, and mRNA transport, as
15 well as DNA damage regulation¹¹² and FUS mutations account for ~5% of familial cases of ALS¹¹³. FUS-
16 related ALS pathology is characterized by mislocalization of FUS to the cytoplasm and a concomitant
17 reduction in nuclear expression in affected neurons. Loss of nuclear FUS leads to neuronal cell death
18 and accumulation of FUS leads to the formation of toxic stress granules¹¹². In their preliminary
19 studies, Zhou and colleagues have demonstrated that FUS is a repressor of its exon 7 and that the
20 exon 7-skipped splice variants of FUS are subjected to nonsense-mediated decay¹¹⁴. A 2'OMe-PS ASO
21 was therefore developed to skip exon 7 and ex-vivo studies demonstrated decrease in FUS protein
22 synthesis and restoration of the deficient FUS autoregulation¹¹⁴.

23

24 Ataxin-2 is an additional target which has been investigated as a therapeutic approach for ALS.
25 Ataxin-2 is an RNA-binding protein involved in RNA metabolism. Presence of high number of CAG
26 trinucleotide repeats in this gene is associated with the severe neurodegenerative disease

1 spinocerebellar ataxia 2 (SCA2) (more than 33 repeats) but also with an increased risk of ALS (29 to
2 33 repeat expansions)¹¹⁵. Reducing expression of ataxin-2 was shown to decrease neurodegeneration
3 in several models, thus opening ASO-mediated therapeutic options to treat these diseases. 2'MOE
4 gapmer ASOs targeting human *ATXN2* were recently screened and shown to decrease *ATXN2* mRNA
5 in cerebella without astroglial and microglial cells activation following ICV injection¹¹⁶. A single
6 treatment markedly extended survival of TDP-43 transgenic mice¹¹⁷, suggesting the therapeutic
7 potential of this approach. Importantly, ASO-mediated reduction of ataxin2 could represent an
8 effective therapeutic strategy for two neurodegenerative diseases, SCA2 and ALS¹¹⁸. It is also
9 important to highlight that it may be beneficial for most forms of ALS since TDP-43 aggregation is a
10 common pathological feature for both sporadic and familial forms of ALS¹¹⁹. However, evidence
11 suggest that ALS related to SOD1-mutations is independent of TDP-43 cascade, implying there would
12 be no benefit with ataxin-2 inhibition¹²⁰.

13

14

15 **6. Huntington's disease**

16 Huntington's disease (HD) is an autosomal dominant neurodegenerative disease characterized by
17 progressive movement dysfunction, cognitive impairment and brain atrophy¹²¹. HD is caused by CAG
18 expansions in the exon 1 of *HTT* gene, encoding an elongated polyglutamine tract in the huntingtin
19 protein (HTT). HTT is critical for nerve cells viability and plays a role in orientation of the mitotic
20 spindle, trafficking of autophagosomes in neurons, and in regulating autophagy¹²². While the
21 pathogenesis of HD is still unclear, most published studies suggest a toxic gain of function for the
22 mutant HTT protein which particularly aggregates and has toxic effects on neurons. Gene silencing
23 approaches including the use of ASOs has been investigated to lower the levels of HTT. ASO therapies
24 for HD offer two main options: the allele specific silencing which aims at knocking down specifically
25 the mutant allele or the non-specific silencing which will lower both alleles and therefore all HTT
26 proteins.

1 **6.1. Non-allele specific approaches**

2 The non-allele specific approach aiming at decreasing the total *HTT* mRNA and protein can be
3 achieved using gapmer ASOs targeting a sequence common to both alleles. Preclinical studies have
4 demonstrated that the ICV infusion of a 2'MOE gapmer ASO during 2 weeks induced efficient and
5 sustainable reduction of HTT in the CNS of YAC128 mice which express the mutant human *HTT*
6 transgene¹²¹. This inhibition of human HTT in mouse models of HD reversed existing behavioral
7 phenotypes and prevented progressive loss of brain mass¹²³. Similar results were also obtained in
8 Rhesus monkeys after intrathecal infusions¹²¹. Based on these encouraging results, a clinical phase
9 1b/2a study was initiated with this 2'MOE gapmer (HTTRx later renamed RG6042) targeting an exonic
10 sequence in the *HTT* RNA. RG6042 was delivered by bolus intrathecal injection every 3 months in 46
11 patients with early stage of HD and five doses were investigated from 10 to 120 mg¹²⁴. The treatment
12 was well-tolerated and a dose-dependent reduction of mutant HTT (mHTT) concentration, which
13 appeared to plateau at higher doses was detected in the CSF¹²⁵. An open-label extension study was
14 started (NCT03342053) as well as a pivotal phase 3 (NCT03761849) at the dose of 120 mg monthly or
15 bimonthly during 25 months to evaluate the long term efficacy and safety of RG6042¹²⁶.

16 **6.2. Allele specific approaches**

17 As previously mentioned and given that HTT is involved in nerve cells viability, there is a concern that
18 non-allele specific approach aiming at lowering total HTT may be detrimental for normal neural
19 function. To circumvent this potential risk, some ASOs have been developed to inhibit specifically the
20 mHTT. One way to preferentially silence the mutant allele is to directly target the CAG repeats which
21 are more represented on the mutant allele. This was achieved in preclinical studies using a steric
22 blocker ASO, fully modified 2'OMe-PS named (CUG)₇. The first result obtained with this ASO *in vitro*
23 confirmed a difference between the inhibition of WT HTT and mHTT with a higher effect with the
24 mutant gene in HD fibroblasts¹²⁷. This difference was confirmed *in vivo* in HD mouse models with a
25 15-60% reduction of both soluble and aggregated mutant HTT protein observed in striatum,
26 hippocampus and cortex of treated mice¹²⁸. In the same study, the authors also investigated the

1 effect of the CUG7 ASO on several endogenous genes with shorter non-expanded CAG repeats and
2 reported no change in expression levels of any of these genes (5 to 20 CAG repeats) suggesting the
3 importance of CAG repeat length for CUG7-mediated silencing¹²⁸.

4 While these encouraging results may suggest an interesting alternative HTT-lowering strategy for HD,
5 this approach has not yet moved to the clinic and the use of CAG targeting ASO is still debated
6 because CAG repeats are also present on the normal allele. Since most patients have a number of
7 CAG repeats relatively close between both alleles, the allele specificity may become more challenging
8 in the clinic. Thus, unambiguous allele-specific approaches have been developed and in particular
9 ASO targeting single-nucleotide polymorphisms (SNP) that are linked to the expanded CAG repeat. 91
10 SNP were found across the *HTT* gene region but the development of only 3 ASOs may enable
11 selective treatment of a maximum of 80% of HD patients¹²⁹. Several preclinical studies using ASO
12 targeting SNPs have demonstrated robust mHTT suppression and the subsequent reduction of
13 cognitive and behavioral impairment in HD mouse models¹³⁰⁻¹³². Wave Live Sciences has been
14 running parallel trials (NCT03225846 and NCT03225833) with two new ASOs, which could
15 theoretically target two-thirds of the HD population; one specific to the region rs362331 (WVE-
16 120101) and the other to rs362307 (WVE-120102). Preliminary results suggest that WVE-120102
17 induced a reduction of 12% of mHTT protein in the CSF of patients treated intrathecally with the
18 highest dose, i.e. 16 mg¹³³. While this figure may appear lower than the previously reported 40-60%
19 reduction of total HTT obtained with RG6042, it is important to note the significant difference in the
20 doses of drug used (16 mg for WVE-120102 vs 120 mg of RG6042). Based on their results showing
21 their drug was safe at lower doses, Wave has also announced it will now add an extra dosing arms to
22 the *PRECISION-HD* trials to test higher doses (32 mg per injection)¹³³.

23 Altogether these results highlight that both allele specific and non-allele specific ASO approaches are
24 moving forward for the treatment for HD and the long term results of these trials will definitely shed
25 some light on the debated role of huntingtin in adults and the safety of a non-allele specific
26 approach.

1

2 **7. CONCLUDING REMARKS**

3 The different examples presented above highlight the variable therapeutic outcomes of the ASO
4 therapies for neuromuscular and neurodegenerative diseases. While some of these drugs have truly
5 demonstrated a significant impact on patient lives, such as the profound clinical benefit observed in
6 SMA patients treated with nusinersen, others have failed to do so. Some of the main reasons for
7 these differences are the target tissue and the route of administration which depend on the disease
8 itself. Indeed the therapeutic outcomes appear much better for diseases where local delivery is
9 possible, as exemplified with SMA, ALS and HD. On the other hand, when the entire musculature
10 must be reached and systemic delivery is the only possible route of administration, things get a bit
11 more complicated, as illustrated by the unsuccessful trial for DM1 and the still limited clinical benefit
12 reported in DMD patients (despite the approval of 3 ASO drugs). Paradoxically the CNS or motor
13 neurons may have been considered more difficult targets to reach than skeletal muscles, but it turns
14 out that ASOs present a relatively good uptake in brain tissues after intrathecal injection.

15 Overall this illustrates more than ever that delivery is key in the success of ASO therapies and that
16 further efforts are needed to improve it. The development of improved targeted delivery is not only
17 required for muscular dystrophies for which systemic administration is needed but would also be
18 advantageous for the diseases currently treated via intrathecal injection. Antisense treatment is
19 administered via intrathecal route because ASO do not cross the BBB, but this way of administration
20 remains an invasive procedure. Besides the obvious clinical challenges, especially when repeated
21 injections are required, it limits the range of applications to severe and rapidly progressive diseases
22 based on the benefit-risk concept. Moreover it neglects the issue of delivering ASOs to the periphery,
23 which could be crucial for some of these diseases like HD or SMA for example^{76,134}. In this context,
24 the development of new ASOs or delivery systems which could deal with the issue of both peripheral
25 and central tissue penetration is also an important milestone.

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Development of more potent chemistries or novel delivery systems has become a very active line of research in the past few years²⁶. Amongst these potentially interesting technological advances was the advent of stereopure ASO chemistry developed by Wave Life sciences, permitting chirally controlled ASO synthesis, with improvements in both potency and safety. However, in spite of having raised hopes in the DMD community, the recent clinical results did not confirm such improved potency and Wave announced the discontinuation of their DMD ASO program. This highlights the caution that is needed in interpreting *in vitro* data, since the apparent 52% of dystrophin restoration measured in human cells *in vitro* vs 1% with the PMO clearly did not translate into the same results in the clinic.

There are many opportunities to further improve the ASO platform and a plethora of delivery technologies are emerging to overcome the very poor intracellular bioavailability of nucleic acid drugs. The recent development (and approval) of ASO incorporating GalNAc (N-Acetylgalactosamine) conjugates showing 20- to 30-fold increases in potency has proven the possibility of creating better antisense drugs. These improvements have impacts on many different levels since increasing potency not only reduces the doses of drug to be used, which may impact the cost, but also reduces or eliminates some of the adverse events often associated with high doses of ASOs.

Inspired by the success of the GalNAc conjugates, the conjugation of ASO to various ligands such as peptides or lipids has been explored. In this context, PPMO have shown very promising results in preclinical studies for DMD⁵⁶, SMA⁸⁰ and DM1⁹⁵. Similarly, lipid-conjugated cEt-ASO have also demonstrated enhanced potency in muscles⁹³. Recently, conjugation of ASOs to antibodies has also gained increasing interest and several groups and companies such as Avidity biosciences have started exploring their potential. However, the promise of all these novel compounds still crucially depends on how well they will be tolerated in humans.

1 Other promising therapeutic approaches have been developed for most of the diseases mentioned in
2 this review and in particular gene replacement therapies mediated by AAV vectors which are
3 particularly useful tools to overcome the delivery challenges in NMDs. AVXS-101, an AAV9 vector
4 designed for *SMN1* replacement was approved by the FDA in 2019 for the treatment of SMA
5 pediatric patients. Given the ability of AAV9 vectors to cross the BBB and the persistence of AAV
6 genomes in tissues, AVXS-101 (commercialized as Zolgensma[®]) offers the advantage of being a
7 single, one-time intravenous (IV) infusion in contrast with nusinersen which is administered
8 intrathecally several times/year.

9 Zolgensma[®] was the first (and only so far) gene therapy drug for SMA and is currently the world's
10 most expensive drug with a price of \$2.1M announced by Novartis. In comparison, spinraza[®]
11 (nusinersen) medication is estimated to cost \$750,000 during the first year of treatment and then
12 \$375,000 annually thereafter ¹³⁵. Gene and ASO therapies are therefore extremely expensive
13 therapies with record high prices, which require a careful consideration of reimbursement and
14 insurance company coverage of medication costs. Nonetheless, it is important to put the costs and
15 benefits of these therapies into perspective, since they offer substantial health gains compared to
16 less expensive conventional treatments and thus may have comparable value in terms of cost-
17 effectiveness ¹³⁶.

18
19 AAV gene therapy programs are also being developed for the treatment of DMD using AAV vectors
20 carrying a microdystrophin transgene. Several clinical trials have been launched to evaluate this
21 strategy in DMD patients and preliminary data have revealed encouraging results with significant
22 restoration of dystrophin expression ¹³⁸. The AAV-microdystrophin approach for DMD offers the
23 advantage of being applicable to most DMD patients as opposed to the personalised ASO therapy
24 targeting specific exons. Yet, the limited packaging capacity of AAV vectors imposes a much shorter
25 dystrophin (microdystrophin) which may turn out less functional than the potential dystrophin
26 resulting from exon skipping. As for the SMA gene therapy, the medication would only require a

1 single IV administration which also compares favourably to the lifelong treatment required with ASO.
2 However, the gene therapy's effectiveness durability still remains to be assessed over long period of
3 time in patients because preclinical studies using AAV vectors in animal models of DMD have
4 suggested a progressive loss of AAV genomes in muscles ^{61,139}. This is of significant importance
5 considering the immunological challenges (such as neutralizing antibodies) faced by the AAV-gene
6 therapy, currently impairing re-administration. In that regard, ASO do not trigger neutralizing
7 antibodies and can therefore be advantageously re-administered. It is thus not excluded to consider
8 future combination therapies of AAV and ASO as it has already been explored in SMA Type 1 patients
9 treated with both Nusinersen and AVXS-101 ¹³⁷.

10

11

12 In conclusion, ASO therapies have made tremendous progress in the past 15 years as illustrated by
13 the numerous approvals of ASOs for the treatment of neuromuscular disorders as well as additional
14 diseases not discussed in this review. Even though many challenges remain, notably with respect to
15 targeted delivery, these first successes have paved the way for future disease modifying-therapies
16 based on the ASO technology.

17

1 **FIGURE LEGENDS**

2

3 **Figure 1: ASOs mechanisms of action.** ASOs may exert different effects depending on their structure
4 and design. To induce mRNA degradation via RNase H, ASO must be designed as gapmers (I) with a
5 central core of 8 to 10 consecutive DNA nucleotides to support binding and cleavage by RNase H1 (in
6 blue), flanked by modified nucleotides for nuclease resistance (in red). Fully modified ASO (II) are not
7 able to elicit RNase H activity and they are commonly used as splice switchers (A) to manipulate
8 alternative splicing (exon skipping or exon inclusion); or as steric blockers (B) to either block protein
9 translation or inhibit RNA mediated toxicity by competing with protein binding. ASO: antisense
10 oligonucleotide.

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14 **TABLES**

15 **Table 1. List of clinical trials for DMD, SMA, DM1, ALS and HD.** Abbreviations: 2'OMe-PS: 2'O-methyl
16 with phosphorothioate link; 2'OMe/ENA: 2'OMe-PS and 2'-O,4'-C-ethylene-bridged nucleic acid
17 chimera ASO; 2'MOE-PS: 2'O-methoxyethyl with phosphorothioate link; ALS : Amyotrophic lateral
18 sclerosis; cET: 2'-4'-constrained ethyl; DM1: Myotonic dystrophy type 1; DMD: Duchenne muscular
19 dystrophy; Ex: exon; HD : Huntington's disease; HTT : Huntington's gene; IM: intramuscular; IV:
20 intravenous; PMO: Phosphorodiamidate Morpholino Oligomer; PPMO: peptide conjugated PMO;
21 SMA: Spinal muscular atrophy; Subcut.: Subcutaneous

22

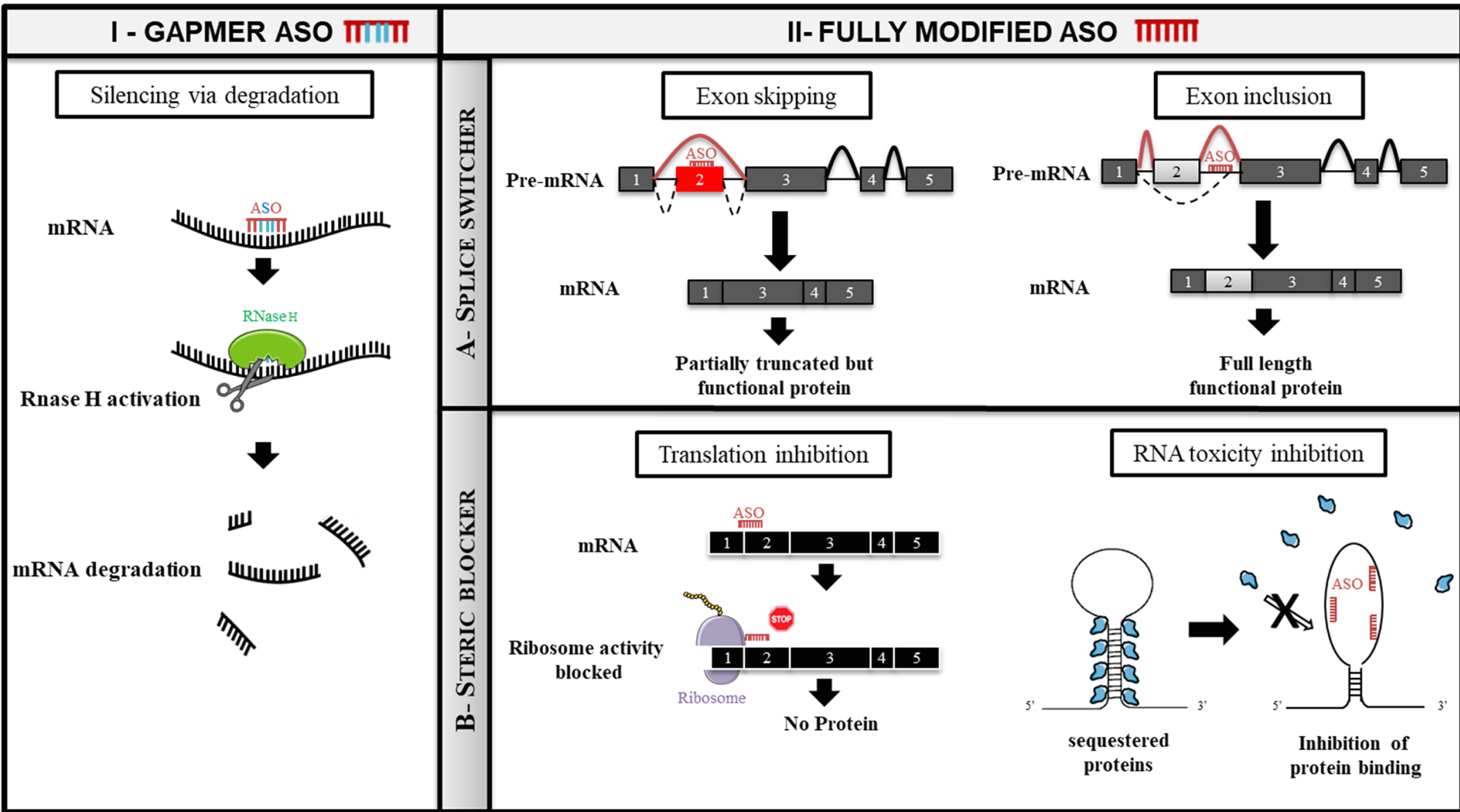
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Figure 1



Disease (target gene)	Drug name	Chemistry and mechanism	Phase	Administration Route	Clinical status	registration number and age of patients	Sponsor	
DMD (Ex20)	AO19	2'OMePS exon skipping	1/2	IV (0.5mg/kg)	completed		Kobe university, Japan	
DMD (Ex51)	PRO051 (Drisapersen®)	2'OMePS exon skipping	1	IM (0.8mg)	completed		Prosensa	
			1/2	Subcut. (escalating doses) and IV	Terminated (2008-2016)	NCT01910649 (5 to 16 years)	Biomarin	
			3	Subcut. and IV (6mg/kg and 3mg/kg)	Terminated (2013-2016)	NCT01803412 (5 years and older)		
				Subcut. (6mg/kg)	Terminated (2011-2014)	NCT01480245 (5 years and older)	GSK	
	Exondys 51® (AVI-4658 / Eteplirsen) Approved by FDA (2016)– rejected by EMA (2018)	PMO exon skipping	1/2	IM (0.09 to 0.9mg)	Completed (2007-2009)	NCT00159250 (10 to 17 years)	Imperial College of London / AVI biopharma	
			1/2	IV (0.5, 1, 2, 4, 10 and 20mg/kg)	Completed (2009-2010)	NCT00844597 (5 to 15 years)	Sarepta	
			2	IV (30 and 50mg/kg)	Completed (2011-2012, 2012-2017, 2014-2018 and 2015-2018)	NCT01396239 and NCT01540409 (7 to 13 years) NCT02286947 (7 to 21 years) NCT02420379 (4 to 6 years)		
			3		Completed (2014-2019)	NCT02255552 (7 to 16 years)		
			3		IV (2 high doses)	Recruiting (2019-2024)		NCT03992430 (7 to 13 years)
			2		IV (2, 4, 10, 20, and 30 mg/kg)	Active, not recruiting (2017-2021)		NCT03218995 (6 to 48Mo babies)
	1	IV (0.5, 1, 2, 5 and 10 mg/kg)	Completed (2018-2019)		NCT03508947 (5 to 18 years)	Wave Life Sciences		
	2/3	IV (2 doses)	Terminated (2019-2020)	NCT03907072 (5 to 12 years)				
	SRP-5051	PPMO exon skipping	1	IV (escalating doses)	Completed (2018-2019)	NCT03375255 (12 years and older)	Sarepta	
			1/2		Recruiting (2018-2024)	NCT03675126 (7 years and older)		
			2	IV (12 or 24 week)	Recruiting (2019-2021)	NCT04004065 (4 to 21 years)		

DMD (Ex44)	PRO044	2'OMePS exon skipping	1/2	Subcut. (0.5, 1.5, 5, 8, 10 and 12 mg/kg) and IV (1.5, 5 and 8 mg/kg)	Completed (2009-2013)	NCT01037309 (5 to 16 years)	Biomarin
			2	Subcut. (6 mg/kg) and IV (6 and 9 mg/kg)	Terminated (2014-2016)	NCT02329769 (9 to 20 years) NCT02958202 (5 years and older)	
DMD (Ex45)	PRO045	2'OMePS exon skipping	1/2	Subcut. (0.15 to 9mg/kg)	Terminated (2013-2016)	NCT01826474 (5 to 18 years)	Biomarin
	SRP-4045 (casimersen®)	PMO exon skipping	1	IV (4 dose levels)	Completed (2015-2018)	NCT02530905 (7 to 21 years)	Sarepta
			3	IV (30mg/kg)	Recruiting (2016-2023)	NCT02500381 (7 to 13 years)	
DS-5141b	2'OMe/ENA exon skipping	1/2	Subcut. (0.1, 0.5, 2 and 6 mg/kg)	Active, not recruiting (2015-2020)	NCT02667483 (5 to 10 years)	Daiichi Sankyo Co.	
DMD (Ex53)	PRO053	2'OMePS exon skipping	2	Subcut. (1, 3, 6 and 9mg/kg) and IV	Terminated (2013-2016)	NCT01957059 (5 to 18 years)	Biomarin
	NS-065/NCNP-01 (Viltolarsen®) Approved in Japan (2020)	PMO exon skipping	1	IV (1.25, 5 and 20 mg/kg)	Completed (2013-2015)	NCT02081625 (5 to 18 years)	National Center of Neurology and Psychiatry, Japan
			2	IV (40 and 80 mg/kg)	Completed (2016-2018)	NCT02740972 (4 to 9 years)	NS Pharma, Inc.
			3	IV (80 mg/kg)	Recruiting (2019-2024)	NCT04060199 (4 to 7 years)	
			2	IV (40 and 80 mg/kg) Child (4 to 10 years old)	Active, not recruiting (2017-2021)	NCT03167255 (4 to 10 years)	
	SRP-4053 (Golodirsen® Vyondys 53®) Approved by FDA (2019)	PMO exon skipping	1/2	IV (4,10,20 and 30 mg/kg)	Completed (2015-2019)	NCT02310906 (6 to 15 years)	Sarepta
3			IV (30 mg/kg)	Recruiting (2016-2023)	NCT02500381 (7 to 13 years)		
SMA	ISIS396443 (Nusinersen®= Spinraza®) Approved by FDA (2016) and EMA (2017)	2'-MOE-PS Exon inclusion	1	Intrathecal (1, 3, 6 and 9mg)	Completed (2011-2013 and 2013-2014)	NCT01494701 NCT01780246 (2-14 years)	Biogen
			1/2	Intrathecal (3, 6, 9 and 12 mg)	Completed (2012-2015)	NCT01703988 (2-15 years)	
			1	Intrathecal (12 mg)	Completed (2014-2017)	NCT02052791 (extension of NCT01703988)	
			2	Intrathecal (6 and 12 mg)	Completed (2013-2017)	NCT01839656 (3 weeks-7 months)	

			2	Intrathecal (9.6 to 12 mg)	Terminated (2015-2018)	NCT02462759 (≤18 months)	
			3	Intrathecal (9.6 to 12 mg)	Active (2015-)	NCT02594124 (extension of NCT02462759)	
			2	Intrathecal (12 mg)	Active (2015-)	NCT02386553 (≤ 6 weeks)	
			3	Intrathecal (2.4 mg)	Terminated (2014- 2016)	NCT02193074 (<7 months old babies)	
			3	Intrathecal (12 mg)	Completed (2014-2017)	NCT02292537 (2-12 years)	
			2	Intrathecal	Recruiting (2018-2022)	NCT03709784 (18 to 70 years)	Washington University School of Medicine
DM1	ISIS-DMPKRx	cET gapmer	1/2	Subcut.	Completed (2014-2016)	NCT02312011	Ionis Pharma.
ALS (SOD1)	BIIB067 (Tofersen®)	2'MOE-PS Gapmer	1	Intrathecal (0.15, 0.5, 1.5 and 3 mg)	Completed (2010-2012)	NCT01041222	Ionis Pharma.
			3	Intrathecal (20, 40, 60, or 100 mg)	Recruiting (2016-2021 and 2017- 2023)	NCT02623699 NCT03070119	Biogen
ALS (C9orf72)	BIIB078 (IONIS-C9Rx®)	2'MOE-PS Gapmer	1	Intrathecal	Active, not recruiting (2018-2021 and 2020- 2023)	NCT03626012 NCT04288856	Biogen
HD (HTT)	RO7234292 = RG6042 = ISIS 443139 = IONIS-HTTRx® (Tominersen®)	2'MOE-PS Gapmer	3	Intrathecal (120mg)	Recruiting (2019 2022)	NCT03761849	Hoffmann- La Roche
	1/2		Intrathecal (10, 30, 60, 90 and 120mg)	Completed (2015-2017)	NCT02519036	Ionis Pharma.	
	WVE-120101 WVE-120102	stereopure Gapmer	1/2	Intrathecal (2, 4, 8, 16 and 32mg)	Recruiting (2017-2020)	NCT03225846 NCT03225833	Wave Life Sciences

1 **COMPLIANCE WITH ETHICAL STANDARDS**

2 **CONFLICT OF INTEREST**

3 AV is an employee of SQY therapeutics, developing tcDNA-ASOs. AG and FB declare no conflict of
4 interest.

5

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10

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11 [releases/news-release-details/wave-life-sciences-announces-suvodirsen-phase-1-safety-and](https://ir.wavelifesciences.com/news-releases/news-release-details/wave-life-sciences-announces-suvodirsen-phase-1-safety-and)>.
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