

Antisense oligonucleotides: the next frontier for treatment of neurological disorders

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Abstract | Despite the discovery that antisense oligonucleotides (ASOs) could influence RNA and modulate protein expression dating back over two decades, the progress of these drugs into the clinic has been hampered by inadequate target engagement, insufficient biological activity and off-target toxic effects. Over the years, novel chemical modifications have been employed to address these issues, which, together with elucidation of the mechanism of action of this new class of drugs, better understanding of disease pathophysiology, and improved clinical trial design have provided momentum for translating ASO-based strategies into therapies. This steady progress recently reached a pinnacle with approvals of the splice-switching ASOs Nusinersen and Eteplirsen for treatment of SMA and DMD respectively, representing a landmark in a field where disease-modifying therapies were virtually non-existent. With the rapid development of improved next-generation ASOs toward clinical application, this technology now holds the potential to have a dramatic impact on the treatment of many neurological and non-neurological conditions in the near future.

Antisense oligonucleotides (ASOs) are short synthetic single-stranded oligodeoxynucleotides, usually 8 to 50 nucleotides in length, which, by pairing to the mRNA target by complementary Watson-Crick base sequences, can alter RNA and reduce, restore, or modify protein expression through several distinct mechanisms. Since the first *in vivo* applications showing limited clinical potential because of the high susceptibility of naked

ASOs to rapid degradation by endo- and exonucleases^{1,2}, ASOs have undergone a large number of chemical modifications leading to improved pharmacological characteristics. The use of a phosphorothioate (PS) backbone, in which one of the non-bridging oxygen atoms is replaced with a sulphur, significantly improved resistance to nuclease activity and increased binding to serum proteins, resulting in longer half-lives in serum³⁻⁵, while still being compatible with applications where target RNA downregulation is desired⁶. Further modifications at the 2' position of the ribose sugar have led to the development of another class of antisense oligonucleotides with improved safety and efficacy profiles. In this group, the 2'-O-methyl (2OMe) and 2'-O-methoxy-ethyl (MOE) are among the most studied. These second-generation antisense agents, containing a PS backbone and 2'-O-substituted oligoribonucleotide segments, have shown increased hybridization affinity to their target RNA⁷⁻⁹, increased resistance towards nuclease degradation^{9,10}, and reduced immunostimulatory activity¹¹ compared with their unmodified counterparts. Other ASOs do not possess the natural phosphate-ribose backbone: in phosphorodiamidate morpholino oligomers (PMO) for example, the deoxyribose moiety is replaced by a morpholine ring, and the charged phosphodiester inter-subunit linkage is replaced by an uncharged phosphorodiamidate linkage¹². These oligonucleotides are very resistant to nuclease and protease degradation¹³ and are mostly exploited in splicing modulation approaches or translation inhibition. Recently two of these second-generation antisense agents have gained FDA approval for spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD), representing a landmark for the field and fuelling unprecedented excitement in this strategy for the treatment of human diseases. In this Review, we timely discuss the properties, applications, and hurdles of antisense pharmacology and the progress made thus far towards clinical applications in neurology.

Pharmacokinetics properties

The evolution of oligonucleotides as therapeutic agents has been hindered by the fact that delivery of these large molecules to their intracellular targets is a very challenging task.

Pharmacokinetic properties of ASOs are similar across species and gender and are largely driven by the chemistry of the backbone^{14–16}. Following systemic administration, plasma concentrations of phosphorothioate-modified ASOs decline in a multi-exponential fashion, where rapid tissue distribution is followed by a slower terminal elimination phase when equilibrium is reached¹⁴. ASOs with a phosphorothioate backbone are extensively associated to plasma proteins with low affinity^{14–16}. Protein binding prevents loss of drug to renal filtration and facilitates tissue uptake, with albumin being the protein that binds the greatest amount across species¹⁷. In contrast, neutrally charged oligonucleotides (e.g. PNAs, morpholinos, and unmodified and unformulated siRNA) bind plasma proteins more weakly and thus are more readily filtered and excreted, resulting in lower tissue uptake¹⁸. It has been well established that where the ASO accumulates in highest concentrations, good antisense activity is routinely observed^{14,19–22}. Systemic administration results in a broad distribution of ASOs into most tissues, particularly liver, kidney, bone marrow, adipocytes, and lymph nodes^{14,15,17,20–23}, and with the notable exception of the central nervous system. In humans, 2OMe ASOs accumulate in proximal tubular cells in the kidney at the highest concentration, therefore requiring careful monitoring of renal function. The blood-brain barrier is largely impervious to oligonucleotides²⁴. However when ASOs are administered by intrathecal injection, they distribute broadly in the central nervous system (CNS), being taken up by both neurons and glial cells both in brain and spinal cord, and with rapid distribution kinetics^{25–29}. It has been demonstrated that, compared to slow infusion, bolus injection into the CSF results in better distribution in the CNS²⁸. Combined with the good clinical safety profile observed to date, intrathecal administration of ASOs holds great potential for application in neurodegenerative diseases^{30–32}.

Intracellular delivery

Intracellular delivery is well recognized as *the* major barrier to effective ASO activity within target cells. While cell uptake is poor, those ASOs that are internalized, are taken up by endocytosis and then traffic to the nucleus, where they encounter their pharmacological

targets. A number of cell-surface receptors have been suggested to bind ASOs, including
 integrins³³, scavenger receptors³⁴, and Toll-like receptors^{35–37}. Studies have shown that
 nuclear entry is not the rate-limiting step for ASO activity, as oligonucleotides, particularly
 those with phosphorothioate backbones, are able to continuously shuttle between the
 nucleus and the cytoplasm, through both passive diffusion and active transport^{38,39}. Whether
 presented in naked form, as a chemical conjugate, or associated with a carrier, an
 oligonucleotide entering a cell encounters an intricate maze of membrane compartments
 which include early and recycling endosomes, late endosomes/multi-vesicular bodies, and
 lysosomes, where the endosomal content is eventually transferred for degradation^{40–42}.
 While the trafficking machinery is usually quite efficient in driving internalized material to the
 appropriate intracellular destination, ASOs must escape the endosome compartments in
 order to reach their target. It is becoming increasingly clear that trafficking of ASOs from
 endosomes into the cytoplasm is a significant rate-limiting step following internalization for
 oligonucleotides therapeutics^{40–42}. Pharmacological interventions aimed at enhancing
 endosomal escape have the potential to improve oligonucleotide activity in the clinic. Current
 strategies rely on altering the endosomal barrier⁴³, modulating intra-endosomal pH using
 titratable peptides or polymers⁴⁴, or selectively permeabilizing the endosomal compartments
 using small molecules to improve oligonucleotide release to the cytosol⁴⁵.

Functional mechanisms

The exact mechanisms by which an oligonucleotide can induce a biological effect are yet to
 be elucidated. Unlike siRNAs, it is likely that ASOs find their targets un-assisted as there
 seems to be no evolved cellular mechanism for promoting antisense strand recognition.
 Once bound to the RNA, ASOs can form an RNA-DNA hybrid that becomes a substrate for
 RNase H, which results in target mRNA degradation⁶. RNase H is a family of ubiquitously
 expressed enzymes that hydrolyses the RNA strand of an RNA/DNA duplex, with RNase H1
 being the necessary mediator⁴⁶ and the rate limiting step for ASO activity⁶. The products of
 the cleaved RNAs are then processed by the normal cellular degradation pathways in both

the nucleus and cytoplasm⁴⁷. ASOs with RNase H competent backbones include the oligodeoxynucleotide phosphodiesteres, the phosphorothioates, and the 2-fluorooligodeoxynucleotides⁴⁸. A minimum stretch of five 2'-deoxy residues is sufficient for RNase H activation *in vitro*^{49,50}. In addition to exploiting cellular nucleases, ASOs can be designed to have intrinsic enzymatic activity, including the ability to directly cleave the target RNA following hybridization (e.g. ribozymes and DNAzymes)^{51,52}. Alternatively, ASOs can modulate gene expression via steric block of the ribosomal machinery⁵³, which can lead to reduced expression, modulation of splicing and/or restoration of a functional protein⁵⁴. Oligonucleotide binding to the pre-mRNA can also be exploited to mask polyadenylation signals on the pre-mRNA, forcing the cell to utilize alternative polyA sites⁵⁵. Other oligonucleotide modifications (2'-O-alkyl, PNA, and morpholinos) may use different mechanisms to inhibit protein expression, e.g. they can inhibit intron excision, a key step in the processing of mRNA. In contrast to RNase H-dependent ASOs, which can inhibit protein expression when hybridised to virtually any transcript, only certain mRNA regions are effective target sites for steric-blocker ASOs.

Proof-of-concept studies for neurological disorders

Splice-switching strategies

The biochemical mechanism of splicing is highly complicated, involving interactions between pre-mRNA, small nuclear ribonucleoproteins and splicing factor proteins and relying on multiple levels of regulation. ASOs hybridizing to splice sites, enhancer or silencer elements within the transcript, allow precise and reproducible manipulation of the splicing machinery, resulting in exon skipping, restoration of a splicing pattern, or shifting the ratio between existing splice forms depending on the designed strategy (FIG. 1). Since the first proof of principle with ASO-mediated splicing correction of the beta-globin transcript⁵⁶, ASOs have emerged as promising tools for the treatment of a number of genetic conditions. In terms of therapeutic development the most advanced use of this technology has been for DMD.

1 *Duchenne muscular dystrophy*. DMD is a severe X-linked myopathy caused by mutations in
2 the *DMD* gene encoding the dystrophin protein. DMD affects about 1/3500-1/4000 live male
3 births globally^{57,58}, thus representing one of the most common fatal genetic diseases^{59,60}.

4 The disease is caused by partial or complete absence of dystrophin, which anchors proteins
5 from the cytoskeleton to those in the myofibre membrane⁶¹, resulting in progressive muscle
6 weakness and atrophy, kyphoscoliosis, cardiomyopathy, and premature death⁶². Analysis of
7 a cohort of over 7000 DMD patients (TREAT-NMD DMD Global database) revealed that the
8 most common mutations lead to the loss of the open reading frame (ORF), with large
9 deletions accounting for 68% of the total mutations, followed by large duplications (11%)⁶³.

10 The first proof-of-concept studies to demonstrate that modulating pre-mRNA splicing of
11 dystrophin using ASOs to restore the dystrophin ORF is a viable therapeutic strategy for
12 DMD were carried out in human lymphoblastoid cells and cultured muscle cells in the mid-
13 1990s⁶⁴⁻⁷¹. The rationale for pursuing an exon-skipping ASO therapy for the treatment of
14 DMD relies on the considerations that the partly shortened protein following internal deletion
15 retains sufficient function to substantially modify the disease course. This is based on the
16 evidence that most of the critical functional domains at the N- and C-terminals within the
17 dystrophin protein are typically unaffected⁷² and that in-frame DMD deletions result in the
18 much milder phenotype of Becker muscular dystrophy. Nevertheless, given the intrinsic
19 nature of the exon-skipping strategy, not all mutations are amenable for correction.

20 Achieving successful restoration of dystrophin expression using ASOs has proven
21 challenging: studies in mdx mice, a mouse model carrying a premature stop codon in exon
22 23 of the *dmd* gene, resulted in 5–6% restoration of dystrophin levels using 2OMe ASOs^{73,74}.

23 Administration of neutrally charged PMO oligonucleotides was able to partially restore levels
24 of the dystrophin protein and ameliorate the disease phenotype in mdx mice⁷⁵⁻⁷⁹. A study
25 comparison between 2OMe and PMO to induce exon skipping in the mdx mouse concluded
26 that PMO resulted in higher levels of dystrophin protein, although other parameters,
27 including the length of the ASO and the identity of the target sequence may likely contribute

to the efficiency of individual oligonucleotides⁷⁵. PMOs have enhanced serum stability, higher resistance to nuclease degradation, a more favourable safety profile, and a wider therapeutic window. In addition, PMOs have shown efficacy in the dog model of DMD. The preclinical studies have raised two main issues of the exon skipping strategy using AONs: the first one being the variability in dystrophin restoration between treated animals and within muscle fibres and the second one related to the restoration of dystrophin expression in non-skeletal muscle and cardiac restoration in particular, which is highly refractory to ASO treatment. This is clinically very relevant because failure to restore dystrophin levels in the heart in mice with restored dystrophin levels in skeletal muscle could exacerbate the cardiac pathology^{76,77}.

Modulation of protein expression

ASOs are often used to downregulate expression of the mutant protein, an approach that has been extensively used in diseases caused by a toxic gain-of-function mechanism, such as Huntington's disease (HD)²⁶ and SOD1 amyotrophic lateral sclerosis (ALS)²⁵. Next to RNase H-mediated degradation, ASO-mediated target suppression can be achieved by blocking translation or splicing modulation to introduce an out-of-frame deletion, which results in protein knockdown by nonsense-mediated decay of the corresponding transcript⁵⁴ (FIG. 1). Increasing the levels of therapeutic proteins *in vivo* is more challenging: approaches such as gene therapy and antisense-mediated de-repression by targeting inhibitory antisense transcripts only gained partial success, due to a number of obstacles, including the limited number of applicable genes^{78–80}. Recently, using a class of modified ASOs that bind to mRNA sequences in upstream open reading frames (uORFs), Liang et al., were able to increase the amounts of protein translated from a downstream primary ORF by 30–150% in a dose dependent manner in both human and mouse cells and by ~80% in mice after systemic treatment⁸¹ (FIG. 1). These findings further broaden the potential utility of ASOs as therapeutic strategies, particularly considering that approximately 50% of human mRNAs have AUGs upstream of the primary start codon^{82,83}.

Huntington's disease. HD is an adult-onset autosomal dominant neurodegenerative condition caused by an abnormal CAG repeat expansion, encoding for a polyglutamine stretch in the HTT protein. This disorder belongs to the family of polyglutamine diseases, which further consists of the spinocerebellar ataxias (SCA 1, 2, 3, 6, 7, and 17), spinal and bulbar muscular atrophy and dentatorubro-pallidoluysian atrophy (Orr H, 2007). The pathological hallmark of these diseases is the accumulation of toxic proteins in affected tissues (Davies, SW 1997; Seidel K, 2012), therefore providing a therapeutic rationale for using an antisense strategy to lower the expression levels of the mutant transcript. Intrathecal infusion of MOE-PS antisense oligonucleotides targeting the human transgene resulted in up to 75% reduction of HTT RNA, extended survival, and improved motor performance in a HD mouse model, up to 8 months post treatment (Kordasiewicz HB, 2012). In late years, in order to limit the detrimental effects of lowering wild-type HTT levels, several allele-specific silencing approaches have been employed, including ASOs targeting the CAG expansion (Hu J, 2009; Gagnon KT, 2010; Hu J, 2010), although they may be associated with the unwanted down-regulation of other transcripts (Sun X, 2014; Hu J, 2009), or single nucleotide polymorphisms (SNPs) enriched on the HD allele (van Bilsen PHJ, 2008; Lombardi MS, 2009; Carroll JB, 2011; Ostergaard ME, 2013). Interestingly, a total of 50 SNPs have been identified on the mutant alleles (Warby SC, 2009). Population genetics studies have shown that 75%–85% of the HD population could be treated using a panel of three to five ASOs targeting these mutant HTT-selective variants (Pfister EL, 2009; Warby SC, 2009).

Tauopathies. Microtubule-associated protein (MAP) tau, MAP1 (A/B) and MAP2 perform similar functions, i.e. the promotion of assembly and stability of the microtubules network in mature neurons. The essential requirement of microtubules for axoplasmic flow, which, in turn, is critical to neuronal activity, may explain this redundancy. The biological activity of tau is regulated by its degree of phosphorylation. Hyperphosphorylation of tau depresses its microtubule assembly activity and its binding to microtubules^{84,85}. In Alzheimer's disease (AD) and related disorders called tauopathies, tau is abnormally hyperphosphorylated and

accumulates into toxic intraneuronal neurofibrillary tangles^{86,87}, an early histopathologic marker⁸⁸ which directly correlates with dementia in these patients^{89–91} and is believed to underlie the widespread neuronal loss⁹². Evidence that mice that completely lack tau only develop a mild motor phenotype later in life^{93–97} and reduction of endogenous tau in adult mice results in no behavioural or neuroanatomical abnormalities have prompted researchers to investigate gene silencing approaches to treat AD and other tauopathies⁹⁸. Morpholinos targeting the start codon, splice acceptors and donors, splicing branch points, polypyrimidine track-related sequences, and splicing enhancer and inhibitor sequences, all resulted in reduction of tau expression in human neuroblastoma cell lines, with the most potent ones inducing skipping of the targeted exons to achieve out-of-frame deletion⁹⁹. Recently treatment of mice overexpressing human tau carrying the P301S mutation¹⁰⁰ with 30 mg/day of a RNase H activating-ASO targeting human tau delivered via intra-cerebroventricular infusion over 28 days was found to significantly reduce tau expression and pathology¹⁰¹. Since by the time symptoms manifest, substantial neuronal loss has already begun in patients, a critical question is whether therapies can slow or even reverse the neurodegenerative process. Importantly, ASO treatment started in aged mice was able to reverse pathological changes and prevent neuronal loss, while improving behavioural deficits, and extending survival¹⁰¹, suggesting that when total human tau is reduced *in vivo*, neurons retain the ability to clear pre-existing neuronal aggregates of tau. The translational potential of this approach is further supported by evidence that in Cynomolgus monkeys delivery of tau-reducing ASO in a single bolus via lumbar puncture into the intrathecal space at doses of 30mg or 50 mg decreased total endogenous tau mRNA in the spinal cord and brain in a dose-dependent manner¹⁰¹. Altogether, *these in vivo* preclinical ASO studies strengthen the case for a tau-reducing therapeutic approach for patients with AD and other tauopathies.

RNA toxicity

1 *Myotonic dystrophy.* Myotonic dystrophy 1 and 2 (DM1 and DM2) are both autosomal
2 dominant neuromuscular conditions caused by an abnormal trinucleotide expansion (CTG)
3 in the 3' UTR of the DMPK (dystrophia myotonica protein kinase) gene (DM1)^{102–106} and a
4 tetranucleotide expansion (CCTG) in the first intron of the ZNF9 (zinc finger 9) gene
5 (DM2)^{104,107–109}, respectively. DM1 and DM2 affect approximately 1 in 8,500 individuals,
6 representing the most common cause of muscular dystrophy in adults^{102,107,110}. The disease
7 mechanism is believed to mainly arise from a RNA toxic gain-of-function where aberrant
8 RNA transcripts containing pathologically expanded (CUG)_n or (CCUG)_n sequences fold
9 into a hairpin-like secondary structure¹¹¹, accumulate in the nucleus and alter the functions
10 of RNA-binding proteins, such as muscleblind-like 1 (MBNL1), involved in regulating mRNA
11 splicing and translation^{112–115}. These alterations cause a global spliceopathy, which results in
12 a multisystemic disorder mainly characterised by myotonia and progressive muscle
13 weakness, cardiac arrhythmias, cataracts and nervous system dysfunction^{116–119}. ASO
14 strategies to treat diseases caused by a toxic RNA can be grouped into two main groups: a)
15 steric hindrance to prevent binding and sequestration of critical RNA-binding proteins, and b)
16 degradation of the mutant transcript by directly targeting the expanded CUG repeat (FIG. 1).
17 CAG25, a PMO antisense oligonucleotide designed to complementary bind with high affinity
18 to the expanded CUG microsatellite repeat region, prevented MBNL1 sequestration,
19 releasing it from the RNA foci, and resulted in at least partial correction of the global
20 misplicing with amelioration of the disease phenotype following intramuscular injection in a
21 DM1 disease model, the HSALR mouse¹²⁰. An alternative strategy involves degradation of
22 the mutant DMPK mRNA using antisense oligonucleotides complementary to a region of the
23 3' UTR that included a (CUG)₁₃ sequence or directly targeting the expanded CUG
24 repeat^{121,122}. This approach using 2OMe antisense oligonucleotides resulted in 90%
25 reduction of DMPK mRNA and improved splicing abnormalities. Importantly, DMPK
26 transcripts containing normal (CUG)_n repeats were largely unaffected.

27 *Amyotrophic lateral sclerosis.* A GGGGCC hexanucleotide repeat expansion in the
28 noncoding region of the C9ORF72 gene accounts for approximately 40% of all inherited

forms of ALS and FTD^{123,124}. The proposed mechanisms of pathogenesis include loss of C9ORF72 protein function, supported by evidence of decreased expression in patients of the repeat-containing allele^{123,125–129}, and RNA toxic gain-of-function arising from folding of repeat-containing RNAs into stable structures, similarly to other non-coding expansion disorders, including myotonic dystrophy^{130,131}. Recent evidence favours the RNA toxic gain-of-function as central in the disease pathogenesis as mice expressing the human C9ORF72 gene with different sizes of expanded repeats develop age- and repeat length-dependent neurological dysfunctions, while mice expressing 50% of C9orf72 mRNA only showed splenomegaly, enlarged lymph nodes, and mild social interaction deficits¹³². Another proposed mechanism of RNA toxicity is the production and accumulation in affected tissues of aberrant dipeptide-repeat (DPR) proteins through repeat-associated non-AUG-dependent (RAN) translation^{133,134}, although their actual contribution to the pathogenesis remains controversial. Irrespective of the relative contribution to neurodegeneration of either RNA-mediated toxicity mechanism, reducing the expanded RNA transcripts without exacerbating a potential loss of C9ORF72 function holds great potential as a therapeutic strategy for this disease. *In vivo* administration of ASOs targeting the C9ORF72 hexanucleotide expansion selectively reduced the repeat-containing RNA levels via a RNase H-dependent mechanism, decreased both soluble and insoluble DPR proteins, and significantly attenuated the behavioural deficits, while preserving exon 1b-containing, C9ORF72 protein-encoding RNAs¹³², confirming previous studies in fibroblasts¹³⁵ and iPS-derived neurons¹³⁶ using a similar strategy.

ASOs en route to the clinic: the SMA experience

The use of ASOs has been known since the '70s; nevertheless, it took several decades to understand the basics of their pharmacology before they could reach the stage of clinical experimentation. Although significant room for improvement still exists, a number of clinical trials have been already completed or are currently underway (Table 1). Among others, the recently approved ASO Nusinersen to treat SMA represents an exemplary case study. SMA

is one of the most prevalent and devastating genetic disorders in childhood¹³⁷. The disease is caused by loss-of-function mutations in a single gene, the survival of motor neuron 1 (*SMN1*) gene¹³⁸. The *SMN2* gene is the primary genetic modifier, since it can generate overall ~10% of the functional protein compared to the *SMN1* locus, the majority of the product lacking exon 7¹³⁹. Discovery of the intronic splicing silencer N1 (ISS-N1), a 15-nucleotide sequence in intron 7 of the *SMN* gene critical for *SMN2* splicing regulation, provided a major breakthrough for the development of an antisense approach in SMA¹⁴⁰. A number of *in vivo* studies, using an optimized ISS-N1-blocking ASO with different modifications, increased the survival and improved the motor phenotype in SMA mouse models^{27,141–143}, and demonstrated a pharmacokinetic advantage of the PMO chemistry over MOE for ICV injections^{144,145}. Preclinical experiments conducted in non-human primates showed that intrathecal infusion of 3mg of ASO over 24 hours was well tolerated and resulted in widespread distribution in the spinal cord. The successful preclinical studies were quickly followed by an open-label phase 1 clinical trial, showing that intrathecal administration of four ascending single-dose levels (1, 3, 6, and 9 mg) of Nusinersen (ISIS-SMNRx) in 28 SMA patients (age 2-14 years) was well-tolerated, resulted in dose-dependent plasma and CSF drug levels, and provided some preliminary evidence of clinical efficacy at the 9 mg dose¹⁴⁶. Following this, a phase 2, open-label, dose-escalation study performed in 20 infants (age 3 weeks and 7 months) where either 6 or 12 mg of Nusinersen were delivered intrathecally on day 1, 15, 85 and 253, with follow up treatments every 4 months, showed mild improvements in motor function at high dose compared to baseline. Importantly, analysis of post-mortem tissue indicated that intrathecal Nusinersen was broadly distributed throughout the spinal cord and brain and that drug concentrations in target motor neurons were above those predicted to produce *SMN2* mRNA exon 7 inclusion³⁰. While no control group was included, results from this trial greatly informed the design of a large phase 3 clinical trial of Nusinersen in infantile-onset SMA, whose results have been recently disclosed. Overall 173 patients were included in the study, 121 as part of a multicentre, randomized, double-blind, sham-controlled investigation (ENDEAR study),

with the remaining included in an open-label study. In an interim analysis conducted on 82 patients treated for at least 183 days, Nusinersen reduced by 47% the risk of death or permanent ventilation in infantile-onset SMA compared to control. In addition, some patients achieved milestones, such as independent sitting (in four cases) and standing (in one), that are almost never observed in the natural history of the disease. These strong results from the interim analysis supported the FDA approval of Nusinersen in the U.S. for the treatment of SMA in paediatric and adult patients, where it will be marketed by Biogen with the name of SPINRAZA™, representing the first approved treatment for individuals with SMA. In October 2016, the European Medicines Agency (EMA) granted Accelerated Assessment status; other countries will follow in 2017.

Future challenges

Recently two ASO-mediated splice switching therapies for SMA and DMD have gained FDA approval, putting neuromuscular diseases at the very forefront of ASO drug development for neurological conditions. These drugs represent the pinnacle of years of steady progress in antisense pharmacology, a field that was virtually non-existent until no more than 2 decades ago. Nevertheless, if these two successful experiences set an example to follow, they also raise a number of issues, which need to be urgently considered in future therapeutic trials using ASOs. In the case of Nusinersen, it remains to be seen whether the efficacy observed in children can be achieved also in adult SMA patients. In addition, the intrathecal delivery does not provide effective SMN correction in peripheral tissues such as muscle, which is known to play a primary role in the disease pathogenesis similarly to other lower motor neuron diseases^{143,147}. Eteplirsen (Exondys 51™), the first drug approved to treat DMD patients, is specifically indicated for patients who have mutations of the dystrophin gene amenable to exon 51 skipping, which represents only about 13 percent of the population with DMD⁶³. Nevertheless, the decision from FDA was based on the surrogate endpoint of a modest 0.9% increase in dystrophin protein in skeletal muscle rather than a clinical benefit in Eteplirsen-treated patients

(<https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm521263.htm>). Thus the future advancement of ASOs in the clinic urgently requires optimization of the following: sequence selection, biological activity, and delivery technology (FIG. 2). These issues are currently being addressed by the field and will hopefully result in better therapeutic efficacy and specificity. Several methods can be employed to improve ASO design, ranging from the more empirical testing of large numbers of mRNA complementary sequences to systematic RNase H mapping, use of combinatorial arrays and of secondary structure prediction by computational methods¹⁴⁸, and in silico pre-screening approach based on predictive statistical modelling¹⁴⁹. In addition, ASOs can achieve allele selective suppression of gene expression by targeting SNPs associated with mutant allele, preserving the function of wild type copy, as it has been shown in models of HD³². Next-generation ASOs with improved pharmacological properties are being tested in animal models and some of them represent promising candidates for clinical testing: one example are tricyclo-DNAs, where an ethylene bridge is fused with a cyclopropane unit, which have been recently reported to promote a higher degree of dystrophin splicing correction in skeletal muscles, heart and brain compared to a 2OMe-PS oligonucleotide following peripheral administration in two DMD mouse models¹⁵⁰. Another recent promising approach to optimise ASO chemistry is centred on the notion that nucleic acid therapeutics consists of a mixture of thousands of stereoisomers; some of them have therapeutic effects, while others are less beneficial or can even contribute to toxic effects. The pharmaceutical company WAVE Life Sciences (Cambridge, MA) has developed a novel chemistry platform to control for ASO chirality. These rationally designed stereopure nucleic acid therapeutics have demonstrated improved activity, stability, specificity and immunogenicity compared with stereoisomer mixtures (Chanda Vargeese: 'Development of Stereopure Nucleic Acid Therapeutics', meeting: Oligonucleotide therapeutics and Delivery. April 2016, Cambridge, MA). Beyond potency and specificity, another critical feature of a good candidate molecule is the ability to reach its intracellular target at sufficient concentration¹⁵¹. An optimal delivery system needs to be cell specific, controllable, and able to protect the nucleic acids from nuclease degradation¹⁵².

Significant progress has been made in recent years in employing lipid- and polymer-based nanocarriers to facilitate antisense delivery¹⁵³. Neutrally charged ASO backbones such as PMO or PNA oligonucleotides conjugated with short cationic peptides, known as cell-penetrating peptides (CPPs) have shown strong transmembrane capacity and great potential for treating neurodegenerative disorders¹⁵⁴. The search for novel CPPs, with a more favourable safety profile and increased efficacy, has led to the identification of a series of peptides known as PNA or PMO internalization peptides (Pips), arisen from structural modifications of an original *Drosophila melanogaster* R6–Penetratin peptide¹⁵⁵. Systemically delivered PPMOs effectively restored therapeutic levels of dystrophin not only in skeletal muscle but also in the heart in animal models^{156–158}. Recently, the advanced peptide-oligonucleotide Pip6a-PMO has demonstrated higher efficacy in both the CNS and peripheral tissues in severe SMA mice following systemic administration than the standard naked ASO¹⁵⁹.

Conclusions

Advances in the understanding of ASO pharmacology, together with the optimization of their efficacy and safety profiles have certainly provided a momentum for translating ASO-based therapeutics into the clinic. The recent approvals of Nusinersen and Eteplirsen for treatment of SMA and DMD respectively will likely pave the way for the use of ASO strategies to treat a wide range of diseases in which the mechanism of disease pathogenesis has been identified. With the growing number of ASO-mediated therapeutics being tested in clinical trials, this technology holds the potential to change the therapeutic landscape for many neurological and non-neurological conditions in the near future.

Contributions

C.R. and M.J.A.W. researched data and wrote the article.

Competing interests statement

C.R. declares no competing interests. M.J.A.W., through the University of Oxford, has filed patents on peptide-based methods for antisense oligonucleotide delivery.

References

1. Dias, N. & Stein, C. A. Antisense oligonucleotides: basic concepts and mechanisms. *Mol. Cancer Ther.* **1**, 347–55 (2002).
2. Eder, P. S., DeVine, R. J., Dagle, J. M. & Walder, J. A. Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3' exonuclease in plasma. *Antisense Res. Dev.* **1**, 141–51 (1991).
3. De Clercq, E., Eckstein, E. & Merigan, T. C. [Interferon induction increased through chemical modification of a synthetic polyribonucleotide]. *Science* **165**, 1137–9 (1969).
4. Rifai, A., Brysch, W., Fadden, K., Clark, J. & Schlingensiepen, K. H. Clearance kinetics, biodistribution, and organ saturability of phosphorothioate oligodeoxynucleotides in mice. *Am. J. Pathol.* **149**, 717–25 (1996).
5. Watanabe, T. A., Geary, R. S. & Levin, A. A. Plasma Protein Binding of an Antisense Oligonucleotide Targeting Human ICAM-1 (ISIS 2302). *Oligonucleotides* **16**, 169–180 (2006).
6. Wu, H. *et al.* Determination of the Role of the Human RNase H1 in the Pharmacology of DNA-like Antisense Drugs. *J. Biol. Chem.* **279**, 17181–17189 (2004).
7. Freier, S. M. & Altmann, K. H. The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA:RNA duplexes. *Nucleic Acids Res.* **25**, 4429–43 (1997).
8. Lubini, P., Zürcher, W. & Egli, M. Stabilizing effects of the RNA 2'-substituent: crystal structure of an oligodeoxynucleotide duplex containing 2'-O-methylated adenosines. *Chem. Biol.* **1**, 39–45 (1994).
9. McKay, R. A. *et al.* Characterization of a potent and specific class of antisense oligonucleotide inhibitor of human protein kinase C- α expression. *J. Biol. Chem.*

- 1 **274**, 1715–22 (1999).
- 2 10. Geary, R. S., Yu, R. Z. & Levin, A. A. Pharmacokinetics of phosphorothioate
3 antisense oligodeoxynucleotides. *Curr. Opin. Investig. Drugs* **2**, 562–73 (2001).
- 4 11. Hamm, S. *et al.* Alternating 2'-O-ribose methylation is a universal approach for
5 generating non-stimulatory siRNA by acting as TLR7 antagonist. *Immunobiology* **215**,
6 559–569 (2010).
- 7 12. SUMMERTON, J. *et al.* Morpholino and Phosphorothioate Antisense Oligomers
8 Compared in Cell-Free and In-Cell Systems. *Antisense Nucleic Acid Drug Dev.* **7**, 63–
9 70 (1997).
- 10 13. HUDZIAK, R. M. *et al.* Resistance of Morpholino Phosphorodiamidate Oligomers to
11 Enzymatic Degradation. *Antisense Nucleic Acid Drug Dev.* **6**, 267–272 (1996).
- 12 14. Geary, R. S. Antisense oligonucleotide pharmacokinetics and metabolism. *Expert*
13 *Opin. Drug Metab. Toxicol.* **5**, 381–391 (2009).
- 14 15. Yu, R. Z., Grundy, J. S. & Geary, R. S. Clinical pharmacokinetics of second
15 generation antisense oligonucleotides. *Expert Opin. Drug Metab. Toxicol.* **9**, 169–82
16 (2013).
- 17 16. Crooke, S. T. & Geary, R. S. Clinical pharmacological properties of mipomersen
18 (Kynamro), a second generation antisense inhibitor of apolipoprotein B. *Br. J. Clin.*
19 *Pharmacol.* **76**, 269–276 (2013).
- 20 17. Levin, A. A., Levine, M. S., Rubesin, S. E. & Laufer, I. An 8-year review of barium
21 studies in the diagnosis of gastroparesis. *Clin. Radiol.* **63**, 407–414 (2008).
- 22 18. Amantana, A. & Iversen, P. L. Pharmacokinetics and biodistribution of
23 phosphorodiamidate morpholino antisense oligomers. *Curr. Opin. Pharmacol.* **5**, 550–
24 5 (2005).
- 25 19. Thompson, J. D. *et al.* Toxicological and pharmacokinetic properties of chemically
26 modified siRNAs targeting p53 RNA following intravenous administration. *Nucleic Acid*
27 *Ther.* **22**, 255–64 (2012).
- 28 20. Yu, R. Z. *et al.* Cross-species comparison of in vivo PK/PD relationships for second-

- 1 generation antisense oligonucleotides targeting apolipoprotein B-100. *Biochem.*
2 *Pharmacol.* **77**, 910–919 (2009).
- 3 21. Zhang, H. *et al.* Reduction of liver Fas expression by an antisense oligonucleotide
4 protects mice from fulminant hepatitis. *Nat. Biotechnol.* **18**, 862–867 (2000).
- 5 22. Altmann, K. H. *et al.* Second-generation antisense oligonucleotides: structure-activity
6 relationships and the design of improved signal-transduction inhibitors. *Biochem. Soc.*
7 *Trans.* **24**, 630–7 (1996).
- 8 23. Hung, G. *et al.* Characterization of Target mRNA Reduction Through *In Situ* RNA
9 Hybridization in Multiple Organ Systems Following Systemic Antisense Treatment in
10 Animals. *Nucleic Acid Ther.* **23**, 369–378 (2013).
- 11 24. Phillips, J. A. *et al.* Pharmacokinetics, metabolism, and elimination of a 20-mer
12 phosphorothioate oligodeoxynucleotide (CGP 69846A) after intravenous and
13 subcutaneous administration. *Biochem. Pharmacol.* **54**, 657–68 (1997).
- 14 25. Smith, R. A. *et al.* Antisense oligonucleotide therapy for neurodegenerative disease.
15 *J. Clin. Invest.* **116**, 2290–2296 (2006).
- 16 26. Kordasiewicz, H. B. *et al.* Sustained Therapeutic Reversal of Huntington's Disease by
17 Transient Repression of Huntingtin Synthesis. *Neuron* **74**, 1031–1044 (2012).
- 18 27. Passini, M. A. *et al.* Antisense Oligonucleotides Delivered to the Mouse CNS
19 Ameliorate Symptoms of Severe Spinal Muscular Atrophy. *Sci. Transl. Med.* **3**,
20 72ra18-72ra18 (2011).
- 21 28. Rigo, F. *et al.* Pharmacology of a Central Nervous System Delivered 2'-O-
22 Methoxyethyl-Modified Survival of Motor Neuron Splicing Oligonucleotide in Mice and
23 Nonhuman Primates. *J. Pharmacol. Exp. Ther.* **350**, 46–55 (2014).
- 24 29. Southwell, A. L., Skotte, N. H., Bennett, C. F. & Hayden, M. R. Antisense
25 oligonucleotide therapeutics for inherited neurodegenerative diseases. *Trends Mol.*
26 *Med.* **18**, 634–643 (2012).
- 27 30. Finkel, R. S. *et al.* Treatment of infantile-onset spinal muscular atrophy with
28 nusinersen: a phase 2, open-label, dose-escalation study. *Lancet* **388**, 3017–3026

(2016).

31. Miller, T. M. *et al.* An antisense oligonucleotide against SOD1 delivered intrathecally for patients with SOD1 familial amyotrophic lateral sclerosis: a phase 1, randomised, first-in-man study. *Lancet Neurol.* **12**, 435–442 (2013).
32. Ostergaard, M. E. *et al.* Rational design of antisense oligonucleotides targeting single nucleotide polymorphisms for potent and allele selective suppression of mutant Huntingtin in the CNS. *Nucleic Acids Res.* **41**, 9634–9650 (2013).
33. Benimetskaya, L. *et al.* Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nat. Med.* **3**, 414–20 (1997).
34. Butler, M., Stecker, K. & Bennett, C. F. Cellular distribution of phosphorothioate oligodeoxynucleotides in normal rodent tissues. *Lab. Invest.* **77**, 379–88 (1997).
35. Robbins, M., Judge, A. & MacLachlan, I. siRNA and innate immunity. *Oligonucleotides* **19**, 89–102 (2009).
36. Kawai, T. & Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity* **34**, 637–650 (2011).
37. Kortylewski, M. *et al.* In vivo delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. *Nat. Biotechnol.* **27**, 925–932 (2009).
38. Lorenz, P., Misteli, T., Baker, B. F., Bennett, C. F. & Spector, D. L. Nucleocytoplasmic shuttling: a novel in vivo property of antisense phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.* **28**, 582–92 (2000).
39. Hartig, R., Shoeman, R. L., Janetzko, A., Grüb, S. & Traub, P. Active nuclear import of single-stranded oligonucleotides and their complexes with non-karyophilic macromolecules. *Biol. cell* **90**, 407–26 (1998).
40. Varkouhi, A. K., Scholte, M., Storm, G. & Haisma, H. J. Endosomal escape pathways for delivery of biologicals. *J. Control. Release* **151**, 220–228 (2011).
41. Juliano, R. L., Ming, X. & Nakagawa, O. Cellular Uptake and Intracellular Trafficking of Antisense and siRNA Oligonucleotides. *Bioconjug. Chem.* **23**, 147–157 (2012).

- 1 42. Juliano, R. L. & Carver, K. Cellular uptake and intracellular trafficking of
2 oligonucleotides. *Adv. Drug Deliv. Rev.* **87**, 35–45 (2015).
- 3 43. Wagenaar, T. R. *et al.* Identification of the endosomal sorting complex required for
4 transport-I (ESCRT-I) as an important modulator of anti-miR uptake by cancer cells.
5 *Nucleic Acids Res.* **43**, 1204–1215 (2015).
- 6 44. Tangsangasaksri, M. *et al.* siRNA-Loaded Polyion Complex Micelle Decorated with
7 Charge-Conversional Polymer Tuned to Undergo Stepwise Response to Intra-
8 Tumoral and Intra-Endosomal pHs for Exerting Enhanced RNAi Efficacy.
9 *Biomacromolecules* **17**, 246–55 (2016).
- 10 45. Yang, B. *et al.* High-throughput screening identifies small molecules that enhance the
11 pharmacological effects of oligonucleotides. *Nucleic Acids Res.* **43**, 1987–1996
12 (2015).
- 13 46. Majorek, K. A. *et al.* The RNase H-like superfamily: new members, comparative
14 structural analysis and evolutionary classification. *Nucleic Acids Res.* **42**, 4160–4179
15 (2014).
- 16 47. Lima, W. F., De Hoyos, C. L., Liang, X. & Crooke, S. T. RNA cleavage products
17 generated by antisense oligonucleotides and siRNAs are processed by the RNA
18 surveillance machinery. *Nucleic Acids Res.* **44**, 3351–3363 (2016).
- 19 48. Deleavey, G. F. & Damha, M. J. Designing chemically modified oligonucleotides for
20 targeted gene silencing. *Chem. Biol.* **19**, 937–54 (2012).
- 21 49. Monia, B. P. *et al.* Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps
22 as antisense inhibitors of gene expression. *J. Biol. Chem.* **268**, 14514–22 (1993).
- 23 50. Wu, H., Lima, W. F. & Crooke, S. T. Properties of cloned and expressed human
24 RNase H1. *J. Biol. Chem.* **274**, 28270–8 (1999).
- 25 51. Breaker, R. R. & Joyce, G. F. A DNA enzyme that cleaves RNA. *Chem. Biol.* **1**, 223–9
26 (1994).
- 27 52. Cech, T. R. & Uhlenbeck, O. C. Ribozymes. Hammerhead nailed down. *Nature* **372**,
28 39–40 (1994).

- 1 53. Baker, B. F. *et al.* 2'-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule
2 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit
3 formation of the ICAM-1 translation initiation complex in human umbilical vein
4 endothelial cells. *J. Biol. Chem.* **272**, 11994–2000 (1997).
- 5 54. Evers, M. M., Toonen, L. J. A. & van Roon-Mom, W. M. C. Antisense oligonucleotides
6 in therapy for neurodegenerative disorders. *Adv. Drug Deliv. Rev.* **87**, 90–103 (2015).
- 7 55. Vickers, T. A., Wyatt, J. R., Burckin, T., Bennett, C. F. & Freier, S. M. Fully modified 2'
8 MOE oligonucleotides redirect polyadenylation. *Nucleic Acids Res.* **29**, 1293–9
9 (2001).
- 10 56. Dominski, Z. & Kole, R. Restoration of correct splicing in thalassemic pre-mRNA by
11 antisense oligonucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8673–7 (1993).
- 12 57. Mah, J. K. *et al.* A systematic review and meta-analysis on the epidemiology of
13 Duchenne and Becker muscular dystrophy. *Neuromuscul. Disord.* **24**, 482–491
14 (2014).
- 15 58. Judge, L. M., Haraguchi, M. & Chamberlain, J. S. Dissecting the signaling and
16 mechanical functions of the dystrophin-glycoprotein complex. *J. Cell Sci.* **119**, 1537–
17 46 (2006).
- 18 59. Parker, A. E. *et al.* Analysis of an adult Duchenne muscular dystrophy population.
19 *QJM* **98**, 729–36 (2005).
- 20 60. Guiraud, S. *et al.* The Pathogenesis and Therapy of Muscular Dystrophies. *Annu.*
21 *Rev. Genomics Hum. Genet.* **16**, 281–308 (2015).
- 22 61. Davies, K. E. & Nowak, K. J. Molecular mechanisms of muscular dystrophies: old and
23 new players. *Nat. Rev. Mol. Cell Biol.* **7**, 762–773 (2006).
- 24 62. Hoffman, E. P., Brown, R. H. & Kunkel, L. M. Dystrophin: the protein product of the
25 Duchenne muscular dystrophy locus. *Cell* **51**, 919–28 (1987).
- 26 63. Bladen, C. L. *et al.* The TREAT-NMD DMD Global Database: Analysis of More than
27 7,000 Duchenne Muscular Dystrophy Mutations. *Hum. Mutat.* **36**, 395–402 (2015).
- 28 64. Dunckley, M. G., Manoharan, M., Villiet, P., Eperon, I. C. & Dickson, G. Modification

- of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligoribonucleotides. *Hum. Mol. Genet.* **7**, 1083–90 (1998).
65. Pramono, Z. A. *et al.* Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence. *Biochem. Biophys. Res. Commun.* **226**, 445–9 (1996).
66. Takeshima, Y., Nishio, H., Sakamoto, H., Nakamura, H. & Matsuo, M. Modulation of in vitro splicing of the upstream intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe. *J. Clin. Invest.* **95**, 515–520 (1995).
67. Aartsma-Rus, A. *et al.* Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. *Neuromuscul. Disord.* **12 Suppl 1**, S71-7 (2002).
68. Aartsma-Rus, A. *et al.* Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. *Gene Ther.* **11**, 1391–1398 (2004).
69. Popplewell, L. J. *et al.* Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human DMD gene: Implications for future clinical trials. *Neuromuscul. Disord.* **20**, 102–110 (2010).
70. Popplewell, L. J., Trollet, C., Dickson, G. & Graham, I. R. Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene. *Mol. Ther.* **17**, 554–61 (2009).
71. van Deutekom, J. C. *et al.* Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum. Mol. Genet.* **10**, 1547–54 (2001).
72. van Deutekom, J. C. T. & van Ommen, G.-J. B. Advances in Duchenne muscular dystrophy gene therapy. *Nat. Rev. Genet.* **4**, 774–783 (2003).
73. Lu, Q. L. *et al.* Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. *Proc. Natl. Acad. Sci.* **102**, 198–203 (2005).

- 1 74. Heemskerk, H. *et al.* Preclinical PK and PD Studies on 2'-O-Methyl-phosphorothioate
2 RNA Antisense Oligonucleotides in the mdx Mouse Model. *Mol. Ther.* **18**, 1210–1217
3 (2010).
- 4 75. Heemskerk, H. A. *et al.* In vivo comparison of 2'-O-methyl phosphorothioate and
5 morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon
6 skipping. *J. Gene Med.* **11**, 257–66 (2009).
- 7 76. Malerba, A., Boldrin, L. & Dickson, G. Long-Term Systemic Administration of
8 Unconjugated Morpholino Oligomers for Therapeutic Expression of Dystrophin by
9 Exon Skipping in Skeletal Muscle: Implications for Cardiac Muscle Integrity. *Nucleic
10 Acid Ther. (Formerly Oligonucleotides)* **21**, 293–298 (2011).
- 11 77. Townsend, D., Yasuda, S., Li, S., Chamberlain, J. S. & Metzger, J. M. Emergent
12 Dilated Cardiomyopathy Caused by Targeted Repair of Dystrophic Skeletal Muscle.
13 *Mol. Ther.* **16**, 832–835 (2008).
- 14 78. Pearson, S., Jia, H. & Kandachi, K. China approves first gene therapy. *Nat.*
15 *Biotechnol.* **22**, 3–4 (2004).
- 16 79. Hoyng, S. A. *et al.* Gene therapy and peripheral nerve repair: a perspective. *Front.*
17 *Mol. Neurosci.* **8**, 32 (2015).
- 18 80. Leader, B., Baca, Q. J. & Golan, D. E. Protein therapeutics: a summary and
19 pharmacological classification. *Nat. Rev. Drug Discov.* **7**, 21–39 (2008).
- 20 81. Liang, X. *et al.* Translation efficiency of mRNAs is increased by antisense
21 oligonucleotides targeting upstream open reading frames. *Nat. Biotechnol.* **34**, 875–
22 880 (2016).
- 23 82. Calvo, S. E., Pagliarini, D. J. & Mootha, V. K. Upstream open reading frames cause
24 widespread reduction of protein expression and are polymorphic among humans.
25 *Proc. Natl. Acad. Sci.* **106**, 7507–7512 (2009).
- 26 83. Lee, S. *et al.* Global mapping of translation initiation sites in mammalian cells at
27 single-nucleotide resolution. *Proc. Natl. Acad. Sci. U. S. A.* **109**, E2424-32 (2012).
- 28 84. Lindwall, G. & Cole, R. D. Phosphorylation affects the ability of tau protein to promote

- 1 microtubule assembly. *J. Biol. Chem.* **259**, 5301–5 (1984).
- 2 85. Alonso, A. C., Zaidi, T., Grundke-Iqbal, I. & Iqbal, K. Role of abnormally
3 phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc.*
4 *Natl. Acad. Sci. U. S. A.* **91**, 5562–6 (1994).
- 5 86. Grundke-Iqbal, I. *et al.* Abnormal phosphorylation of the microtubule-associated
6 protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. U. S. A.*
7 **83**, 4913–7 (1986).
- 8 87. Iqbal, K. *et al.* Defective brain microtubule assembly in Alzheimer's disease. *Lancet*
9 *(London, England)* **2**, 421–6 (1986).
- 10 88. Braak, H. & Braak, E. Neuropathological stageing of Alzheimer-related changes. *Acta*
11 *Neuropathol.* **82**, 239–59 (1991).
- 12 89. Tomlinson, B. E., Blessed, G. & Roth, M. Observations on the brains of demented old
13 people. *J. Neurol. Sci.* **11**, 205–42 (1970).
- 14 90. Alafuzoff, I., Iqbal, K., Friden, H., Adolfsson, R. & Winblad, B. Histopathological
15 criteria for progressive dementia disorders: clinical-pathological correlation and
16 classification by multivariate data analysis. *Acta Neuropathol.* **74**, 209–25 (1987).
- 17 91. Arriagada, P. V, Growdon, J. H., Hedley-Whyte, E. T. & Hyman, B. T. Neurofibrillary
18 tangles but not senile plaques parallel duration and severity of Alzheimer's disease.
19 *Neurology* **42**, 631–9 (1992).
- 20 92. Hyman, B. T., Van Hoesen, G. W., Damasio, A. R. & Barnes, C. L. Alzheimer's
21 disease: cell-specific pathology isolates the hippocampal formation. *Science* **225**,
22 1168–70 (1984).
- 23 93. Lei, P. *et al.* Tau deficiency induces parkinsonism with dementia by impairing APP-
24 mediated iron export. *Nat. Med.* **18**, 291–295 (2012).
- 25 94. Morris, M. *et al.* Age-appropriate cognition and subtle dopamine-independent motor
26 deficits in aged tau knockout mice. *Neurobiol. Aging* **34**, 1523–9 (2013).
- 27 95. Li, Z., Hall, A. M., Kelinske, M. & Roberson, E. D. Seizure resistance without
28 parkinsonism in aged mice after tau reduction. *Neurobiol. Aging* **35**, 2617–2624

- 1 (2014).
- 2 96. Roberson, E. D. *et al.* Reducing Endogenous Tau Ameliorates Amyloid -Induced
3 Deficits in an Alzheimer's Disease Mouse Model. *Science* (80-.). **316**, 750–754
4 (2007).
- 5 97. van Hummel, A. *et al.* No Overt Deficits in Aged Tau-Deficient
6 C57Bl/6.Mapt^{tm1(EGFP)Kit} GFP Knockin Mice. *PLoS One* **11**, e0163236 (2016).
- 7 98. DeVos, S. L. & Miller, T. M. Antisense oligonucleotides: treating neurodegeneration at
8 the level of RNA. *Neurotherapeutics* **10**, 486–97 (2013).
- 9 99. Sud, R., Geller, E. T. & Schellenberg, G. D. Antisense-mediated Exon Skipping
10 Decreases Tau Protein Expression: A Potential Therapy For Tauopathies. *Mol. Ther. -*
11 *Nucleic Acids* **3**, e180 (2014).
- 12 100. Yoshiyama, Y. *et al.* Synapse loss and microglial activation precede tangles in a
13 P301S tauopathy mouse model. *Neuron* **53**, 337–51 (2007).
- 14 101. DeVos, S. L. *et al.* Tau reduction prevents neuronal loss and reverses pathological
15 tau deposition and seeding in mice with tauopathy. *Sci. Transl. Med.* **9**, eaag0481
16 (2017).
- 17 102. Jiang, H., Mankodi, A., Swanson, M. S., Moxley, R. T. & Thornton, C. A. Myotonic
18 dystrophy type 1 is associated with nuclear foci of mutant RNA, sequestration of
19 muscleblind proteins and deregulated alternative splicing in neurons. *Hum. Mol.*
20 *Genet.* **13**, 3079–88 (2004).
- 21 103. Mahadevan, M. S. *et al.* Reversible model of RNA toxicity and cardiac conduction
22 defects in myotonic dystrophy. *Nat. Genet.* **38**, 1066–1070 (2006).
- 23 104. Ranum, L. P. W. & Cooper, T. A. RNA-MEDIATED NEUROMUSCULAR
24 DISORDERS. *Annu. Rev. Neurosci.* **29**, 259–277 (2006).
- 25 105. Orengo, J. P. *et al.* Expanded CTG repeats within the DMPK 3' UTR causes severe
26 skeletal muscle wasting in an inducible mouse model for myotonic dystrophy. *Proc.*
27 *Natl. Acad. Sci. U. S. A.* **105**, 2646–51 (2008).
- 28 106. Yadava, R. S. *et al.* RNA toxicity in myotonic muscular dystrophy induces NKX2-5

- expression. *Nat. Genet.* **40**, 61–8 (2008).
107. Day, J. W. *et al.* Myotonic dystrophy type 2: molecular, diagnostic and clinical spectrum. *Neurology* **60**, 657–64 (2003).
108. Liquori, C. L. *et al.* Myotonic Dystrophy Type 2: Human Founder Haplotype and Evolutionary Conservation of the Repeat Tract. *Am. J. Hum. Genet.* **73**, 849–862 (2003).
109. Margolis, J. M., Schoser, B. G., Moseley, M. L., Day, J. W. & Ranum, L. P. W. DM2 intronic expansions: evidence for CCUG accumulation without flanking sequence or effects on ZNF9 mRNA processing or protein expression. *Hum. Mol. Genet.* **15**, 1808–1815 (2006).
110. Lee, J. E. & Cooper, T. A. Pathogenic mechanisms of myotonic dystrophy. *Biochem. Soc. Trans.* **37**, 1281–1286 (2009).
111. Napierała, M. & Krzyzosiak, W. J. CUG repeats present in myotonin kinase RNA form metastable “slippery” hairpins. *J. Biol. Chem.* **272**, 31079–85 (1997).
112. Kuyumcu-Martinez, N. M. & Cooper, T. A. Misregulation of alternative splicing causes pathogenesis in myotonic dystrophy. *Prog. Mol. Subcell. Biol.* **44**, 133–59 (2006).
113. Cardani, R., Mancinelli, E., Rotondo, G., Sansone, V. & Meola, G. Muscleblind-like protein 1 nuclear sequestration is a molecular pathology marker of DM1 and DM2. *Eur. J. Histochem.* **50**, 177–82
114. Fardaei, M., Larkin, K., Brook, J. D. & Hamshire, M. G. In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts. *Nucleic Acids Res.* **29**, 2766–71 (2001).
115. Fardaei, M. *et al.* Three proteins, MBNL, MBLL and MBXL, co-localize in vivo with nuclear foci of expanded-repeat transcripts in DM1 and DM2 cells. *Hum. Mol. Genet.* **11**, 805–14 (2002).
116. Ward, A. J., Rimer, M., Killian, J. M., Dowling, J. J. & Cooper, T. A. CUGBP1 overexpression in mouse skeletal muscle reproduces features of myotonic dystrophy type 1. *Hum. Mol. Genet.* **19**, 3614–3622 (2010).

- 1 117. Yuan, Y. *et al.* Muscleblind-like 1 interacts with RNA hairpins in splicing target and
2 pathogenic RNAs. *Nucleic Acids Res.* **35**, 5474–86 (2007).
- 3 118. Koshelev, M., Sarma, S., Price, R. E., Wehrens, X. H. T. & Cooper, T. A. Heart-
4 specific overexpression of CUGBP1 reproduces functional and molecular
5 abnormalities of myotonic dystrophy type 1. *Hum. Mol. Genet.* **19**, 1066–1075 (2010).
- 6 119. Du, H. *et al.* Aberrant alternative splicing and extracellular matrix gene expression in
7 mouse models of myotonic dystrophy. *Nat. Struct. Mol. Biol.* **17**, 187–93 (2010).
- 8 120. Wheeler, T. M. *et al.* Reversal of RNA Dominance by Displacement of Protein
9 Sequestered on Triplet Repeat RNA. *Science (80-.)*. **325**, 336–339 (2009).
- 10 121. Furling, D. *et al.* Viral vector producing antisense RNA restores myotonic dystrophy
11 myoblast functions. *Gene Ther.* **10**, 795–802 (2003).
- 12 122. Mulders, S. A. M. *et al.* Triplet-repeat oligonucleotide-mediated reversal of RNA
13 toxicity in myotonic dystrophy. *Proc. Natl. Acad. Sci.* **106**, 13915–13920 (2009).
- 14 123. DeJesus-Hernandez, M. *et al.* Expanded GGGGCC Hexanucleotide Repeat in
15 Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS.
16 *Neuron* **72**, 245–256 (2011).
- 17 124. Renton, A. E. *et al.* A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of
18 Chromosome 9p21-Linked ALS-FTD. *Neuron* **72**, 257–268 (2011).
- 19 125. Belzil, V. V, Gendron, T. F. & Petrucelli, L. RNA-mediated toxicity in
20 neurodegenerative disease. *Mol. Cell. Neurosci.* **56**, 406–19 (2013).
- 21 126. Gijselinck, I. *et al.* A C9orf72 promoter repeat expansion in a Flanders-Belgian cohort
22 with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis
23 spectrum: a gene identification study. *Lancet. Neurol.* **11**, 54–65 (2012).
- 24 127. Liu, E. Y. *et al.* C9orf72 hypermethylation protects against repeat expansion-
25 associated pathology in ALS/FTD. *Acta Neuropathol.* **128**, 525–541 (2014).
- 26 128. Xi, Z. *et al.* Hypermethylation of the CpG Island Near the G4C2 Repeat in ALS with a
27 C9orf72 Expansion. *Am. J. Hum. Genet.* **92**, 981–989 (2013).
- 28 129. Xi, Z. *et al.* The C9orf72 repeat expansion itself is methylated in ALS and FTLD

- patients. *Acta Neuropathol.* **129**, 715–727 (2015).
130. Gendron, T. F., Belzil, V. V., Zhang, Y.-J. & Petrucelli, L. Mechanisms of toxicity in C9FTLD/ALS. *Acta Neuropathol.* **127**, 359–376 (2014).
131. Ling, S.-C., Polymenidou, M. & Cleveland, D. W. Converging Mechanisms in ALS and FTD: Disrupted RNA and Protein Homeostasis. *Neuron* **79**, 416–438 (2013).
132. Jiang, J. *et al.* Gain of Toxicity from ALS/FTD-Linked Repeat Expansions in C9ORF72 Is Alleviated by Antisense Oligonucleotides Targeting GGGGCC-Containing RNAs. *Neuron* **90**, 535–550 (2016).
133. Zu, T. *et al.* Non-ATG-initiated translation directed by microsatellite expansions. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 260–5 (2011).
134. Cleary, J. D. & Ranum, L. P. W. Repeat-associated non-ATG (RAN) translation in neurological disease. *Hum. Mol. Genet.* **22**, R45–R51 (2013).
135. Lagier-Tourenne, C. *et al.* Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. *Proc. Natl. Acad. Sci.* **110**, E4530–E4539 (2013).
136. Donnelly, C. J. *et al.* RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. *Neuron* **80**, 415–28 (2013).
137. Prior, T. W. & Finanger, E. *Spinal Muscular Atrophy. GeneReviews®* (1993). at <<http://www.ncbi.nlm.nih.gov/pubmed/20301526>>
138. Brzustowicz, L. M. *et al.* Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q1 1.2–13.3. *Nature* **344**, 540–541 (1990).
139. Burghes, A. H. M. & Beattie, C. E. Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? *Nat. Rev. Neurosci.* **10**, 597–609 (2009).
140. Singh, N. K., Singh, N. N., Androphy, E. J. & Singh, R. N. Splicing of a critical exon of human Survival Motor Neuron is regulated by a unique silencer element located in the last intron. *Mol. Cell. Biol.* **26**, 1333–46 (2006).
141. Williams, J. H. *et al.* Oligonucleotide-Mediated Survival of Motor Neuron Protein

- Expression in CNS Improves Phenotype in a Mouse Model of Spinal Muscular Atrophy. *J. Neurosci.* **29**, 7633–7638 (2009).
142. Hua, Y. *et al.* Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev.* **24**, 1634–44 (2010).
143. Hua, Y. *et al.* Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* **478**, 123–126 (2011).
144. Porensky, P. N. *et al.* A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse. *Hum. Mol. Genet.* **21**, 1625–38 (2012).
145. Zhou, H. *et al.* A Novel Morpholino Oligomer Targeting ISS-N1 Improves Rescue of Severe Spinal Muscular Atrophy Transgenic Mice. *Hum. Gene Ther.* **24**, 331–342 (2013).
146. Chiriboga, C. A. *et al.* Results from a phase 1 study of nusinersen (ISIS-SMN_{Rx}) in children with spinal muscular atrophy. *Neurology* **86**, 890–897 (2016).
147. Boyer, J. G., Ferrier, A. & Kothary, R. More than a bystander: the contributions of intrinsic skeletal muscle defects in motor neuron diseases. *Front. Physiol.* **4**, 356 (2013).
148. Smith, L., Andersen, K. B., Hovgaard, L. & Jaroszewski, J. W. Rational selection of antisense oligonucleotide sequences. *Eur. J. Pharm. Sci.* **11**, 191–8 (2000).
149. Echigoya, Y., Mouly, V., Garcia, L., Yokota, T. & Duddy, W. In Silico Screening Based on Predictive Algorithms as a Design Tool for Exon Skipping Oligonucleotides in Duchenne Muscular Dystrophy. *PLoS One* **10**, e0120058 (2015).
150. Goyenvalle, A. *et al.* Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. *Nat. Med.* (2015). doi:10.1038/nm.3765
151. Alavijeh, M. S., Chishty, M., Qaiser, M. Z. & Palmer, A. M. Drug metabolism and pharmacokinetics, the blood-brain barrier, and central nervous system drug discovery. *NeuroRx* **2**, 554–71 (2005).
152. Bedi, D. *et al.* Targeted Delivery of siRNA into Breast Cancer Cells via Phage Fusion Proteins. *Mol. Pharm.* **10**, 551–559 (2013).

- 1 153. Falzarano, M. S., Passarelli, C. & Ferlini, A. Nanoparticle Delivery of Antisense
2 Oligonucleotides and Their Application in the Exon Skipping Strategy for Duchenne
3 Muscular Dystrophy. *Nucleic Acid Ther.* **24**, 87–100 (2014).
- 4 154. Lehto, T., Ezzat, K., Wood, M. J. A. & EL Andaloussi, S. Peptides for nucleic acid
5 delivery. *Adv. Drug Deliv. Rev.* **106**, 172–182 (2016).
- 6 155. Ivanova, G. D. *et al.* PNA-peptide conjugates as intracellular gene control agents.
7 *Nucleic Acids Symp. Ser.* **52**, 31–32 (2008).
- 8 156. Jearawiriyapaisarn, N. *et al.* Sustained dystrophin expression induced by peptide-
9 conjugated morpholino oligomers in the muscles of mdx mice. *Mol. Ther.* **16**, 1624–9
10 (2008).
- 11 157. Wu, B. *et al.* Effective rescue of dystrophin improves cardiac function in dystrophin-
12 deficient mice by a modified morpholino oligomer. *Proc. Natl. Acad. Sci. U. S. A.* **105**,
13 14814–9 (2008).
- 14 158. Yin, H. *et al.* Pip5 Transduction Peptides Direct High Efficiency Oligonucleotide-
15 mediated Dystrophin Exon Skipping in Heart and Phenotypic Correction in mdx Mice.
16 *Mol. Ther.* **19**, 1295–1303 (2011).
- 17 159. Hammond, S. M. *et al.* Systemic peptide-mediated oligonucleotide therapy improves
18 long-term survival in spinal muscular atrophy. *Proc. Natl. Acad. Sci.* **113**, 10962–
19 10967 (2016).
- 20 160. Goemans, N. M. *et al.* Systemic administration of PRO051 in Duchenne’s muscular
21 dystrophy. *N. Engl. J. Med.* **364**, 1513–1522 (2011).
- 22 161. Voit, T. *et al.* Safety and efficacy of drisapersen for the treatment of Duchenne
23 muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled
24 phase 2 study. *Lancet. Neurol.* **13**, 987–996 (2014).
- 25 162. Kinali, M. *et al.* Local restoration of dystrophin expression with the morpholino
26 oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-
27 controlled, dose-escalation, proof-of-concept study. *Lancet Neurol.* **8**, 918–928
28 (2009).

163. Cirak, S. *et al.* Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet* **378**, 595–605 (2011).
164. Mendell, J. R. *et al.* Eteplirsen for the treatment of Duchenne muscular dystrophy. *Ann. Neurol.* **74**, 637–647 (2013).

Figure legends

Figure 1. Functional mechanisms of antisense oligonucleotide. **A** | Once bound to the RNA, ASOs can form an RNA-DNA hybrid that becomes a substrate for RNase H, which results in target mRNA degradation. **B** | ASOs targeting the AUG start site can sterically block the binding of RNA binding protein complexes, such as ribosomal subunits, suppressing translation of target mRNA. **C** | In diseases caused by a RNA toxic gain-of-function mechanism, antisense designed to complementary bind with high affinity untranslated regions can prevent binding and sequestration of RNA-binding proteins by steric hindrance. **D** | ASOs binding to splice sites or exonic/intronic inclusion signals results in skipping or inclusion of the targeted exon. **E** | Translation of the upstream open reading frames (uORFs) typically inhibits the expression of the primary ORF. ASOs binding to the uORFs are able to increase the amounts of protein translated from a downstream ORF.

Figure 2. Development of next-generation oligonucleotides. Advancement of ASOs as a viable therapeutic approach for human diseases urgently requires further optimization of sequence selection, chemistry and delivery technology. This schematic figure shows the most recent strategies adopted in the development of next-generation ASOs. **A** | ASO potency and specificity can be improved by a more rational sequence selection, based on empirical testing and use of predictive modelling, and targeting allele-specific single

1 nucleotide polymorphisms. **B** | Modification of ASO chemistry, such as the most recent
2 tricyclo-DNAs, phosphoryl guanidine oligo(2'-OMe)ribonucleotides (PGO), and use of
3 stereopure chemistry, can result in more efficient target engagement and reduced toxicity *in*
4 *vivo*. **C** | One of the major hurdles that severely limit clinical application of ASOs is low
5 intracellular delivery. Strategies to improve it include targeted ligand-oligonucleotide
6 conjugates, lipid- and polymer-based nanoparticles, antibody conjugates and use of cell-
7 penetrating peptides.

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