




# Antisense Oligonucleotides for the Study and Treatment of ALS

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## Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by motor neuron loss. ALS is now associated with mutations in numerous genes, many of which cause disease in part through toxic gain-of-function mechanisms. Antisense oligonucleotides (ASOs) are small sequences of DNA that can reduce expression of a target gene at the post-transcriptional level, making them attractive for neutralizing mutant or toxic gene products. Advancements in the medicinal chemistries of ASOs have improved their pharmacodynamic profile to allow safe and effective delivery to the central nervous system. ASO therapies for ALS have rapidly developed over the last two decades, and ASOs that target *SOD1*, *C9orf72*, *FUS*, and *ATXN2* are now in clinical trials for familial or sporadic forms of ALS. This review discusses the current state of ASO therapies for ALS, outlining their successes from preclinical development to early clinical trials.

**Keywords** Amyotrophic lateral sclerosis · Antisense oligonucleotide · Clinical trials · Therapy · Motor neuron disease

## Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing, devastating neurodegenerative disease that causes motor neuron loss and muscle atrophy. Most cases of ALS occur sporadically, whereas 10% are familial (fALS) and can usually be attributed to a single dominantly inherited genetic risk factor [1]. Patients with a monogenic cause of ALS establish a population that shares a common etiology and an apparent druggable target [1]. Since the identification of mutations in copper-zinc superoxide dismutase 1 (*SOD1*) as the first genetic cause of fALS [2], advancements in genome technologies have linked mutations in > 30 genes to ALS pathogenesis [3]. It is now recognized that most fALS cases can be attributed to a hexanucleotide expansion in the first intron of chromosome 9 open reading frame 72 (*C9orf72*) [4–6]. Decades of mechanistic studies have revealed that many monogenic causes of ALS occur in part through toxic gain of function (GOF) by the mutated gene [7]. For these cases, emerging therapeutic strategies that directly alter

disease-causing genes and can neutralize mutant or toxic gene products carry immense promise.

Antisense oligonucleotides (ASOs) enable precise modulation of gene expression without altering host DNA, a limitation of current gene transfer approaches. ASOs modify protein expression by acting directly on target RNA either prior to or during translation [8]. Owing to their efficacy, ASOs have been powerful tools for mechanistic investigation for decades. Recent advancements in the medicinal chemistries of ASOs have elevated ASOs from investigational tools to safe, tolerable, and efficacious drugs with clinical utility [8]. ASOs are now approved for the treatment of > 10 conditions that include spinal muscular atrophy (SMA), a pediatric-onset motor neuron disease [9]. For diseases with a pathogenesis tied to single gene mutations or mechanisms, ASOs have become a top consideration, owing to straightforward validation in disease model systems and to demonstrated successes in clinical translation.

Cellular and animal model systems that phenotypically and pathologically resemble ALS have facilitated ASO drug advancement. Skin biopsies and peripheral blood cells from patients carrying disease mutations have allowed ASO testing in patient-derived cells or reprogrammed induced pluripotent stem cell (iPSC) lines. Remarkably, these models phenocopy many cellular features of disease and are now a mainstay in the first phase of ASO drug development. Candidate ASOs with convincing on-target action and efficacy have since advanced to clinical trials. Here, we will discuss

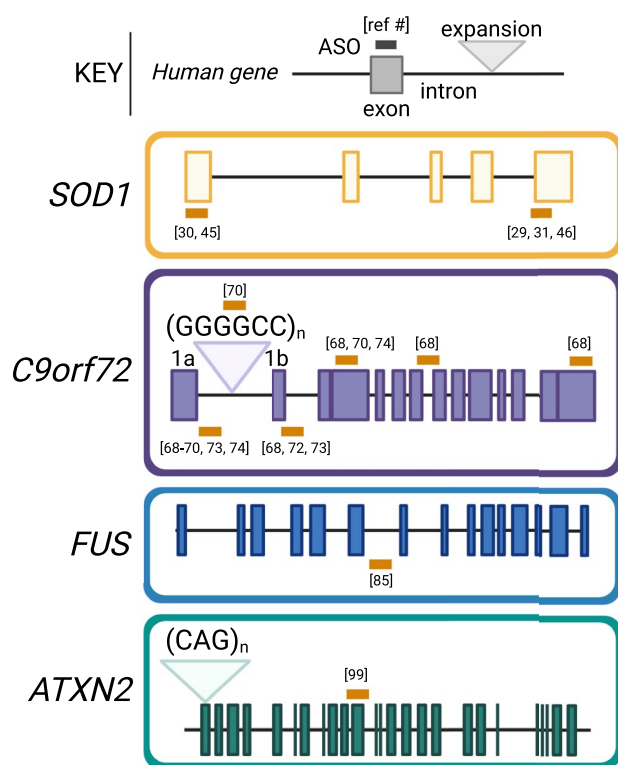
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advances and challenges in ASO technologies for *SOD1*, *C9orf72*, *FUS*, and *ATXN2* (Fig. 1) and the pipelines that have elevated each from ASO discovery to human clinical trials for ALS.

## Antisense Oligonucleotide Design and Mechanisms

ASOs are single-stranded DNA oligomers of 10–50 base pairs in length that bind to a target RNA through Watson-Crick-Franklin base pairing. Native, unmodified oligonucleotides have weak binding affinities and are highly susceptible to nucleases. However, modifications to the phosphate backbone or sugar rings confer favorable binding affinity and stability to ASOs and define their mechanisms of action [10].



**Fig. 1** Antisense oligonucleotide therapies under investigation in pre-clinical studies and human clinical trials for amyotrophic lateral sclerosis. ASO candidates targeting genes associated with ALS, including *SOD1* [29–31, 45, 46], *C9orf72* [68–70, 72–74], *FUS* [85], and *ATXN2* [99], have demonstrated target efficacy, distribution throughout the central nervous system, and a strong safety profile. ASOs depicted target the human form of the gene and have been tested in preclinical and/or clinical trials (see references for more detail). ASO depictions do not represent exact binding locations. Note: the binding site of WVE-004 within the *C9orf72* gene [71] is not included in this figure. Figure created with BioRender.com

## ASO Chemistry and Modifications

One of the most common ASO chemistries involves modifications to the phosphodiester linkages forming the phosphate backbone [10]. Here, the substitution of the non-bridging phosphate oxygen atom for sulfur creates a phosphorothioate (PS) backbone that confers drug-like properties of many ASOs. First, the PS backbone promotes ASO stability and affords greater nuclease resistance, increasing the likelihood for target RNA engagement [10]. Second, the PS backbone can recruit the enzyme RNase H to cleave a target transcript, conferring PS-ASOs with degrading capability [10].

In addition to backbone modifications, ASOs can be modified at the 2'-position of the ribose moiety to further increase stability and binding [10]. Most used and represented in clinical trials are the 2'-O-methyl (2'-O-Me) and 2'-O-methoxyethyl (2'-MOE) modifications, which strengthen binding affinity to its target and promote nuclease resistance [9, 11]. Importantly, inclusion of 2'-O-Me or 2'-MOE bases reduces immune stimulation, which might occur due to PS use [12–14]. ASOs with “fully modified” sugar rings, in which all bases contain a 2' modification, support non-degrading ASO mechanisms such as splicing manipulations but do not support RNase H enzymatic activity. However, a “gapmer” ASO design — in which 2'-O-Me or 2'-MOE-modified bases flank a central “gap” of unmodified PS bases — can degrade target RNA but maintain a low immunostimulatory profile [9, 11, 13, 15].

Recent innovations in ASO design include mixed backbone ASOs with varying combinations of base modifications. One interesting hypothesis is that creating ASOs with a predefined stereochemistry might improve potency by stabilizing 2' modifications and enhancing RNase H recruitment, and some of these ASOs are currently in clinical trials [11, 16]. Other modifications to the backbone or ribose sugar, such as morpholinos or locked nucleic acid (LNA) formulations, have great potential and are also progressing through clinical trials [17, 18]. Morpholinos have high binding affinity but do not recruit RNase H [10]. LNAs are a variant on 2'-O-Me structures with superior binding affinity and nuclease resistance that have been limited due to potential toxicity profile, though new formulations might mitigate some concerns [10, 19–21]. Designing ASOs with diverse backbone types has become popular in recent years, but whether these modifications improve ASO function or attenuate toxicity over unmixed formulations is still not known.

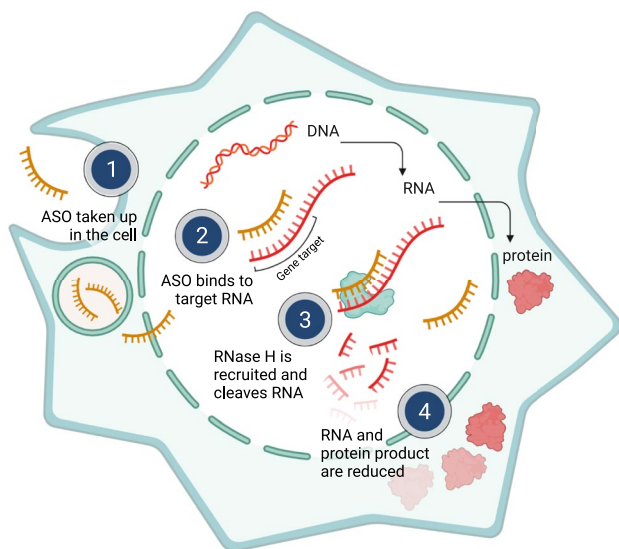
## Mechanisms of Action

The mechanism of action for an ASO depends on its sequence and design. Once an ASO enters a cell, it can readily translocate

to the nucleus, where it binds complementarily to target RNA. The ASO sequence confers extremely high target specificity, and its presence in the nucleus allows target engagement at the earliest stages of processing [15]. Here, backbone and 2' sugar modifications determine the activity of an ASO, which we divide into RNase-mediated degrading or non-degrading mechanisms [10].

ASOs working through RNase H-dependent cleavage mechanisms (Fig. 2) are most commonly used in pre-clinical testing and in clinical trials for ALS. RNase H responds to RNA–DNA heteroduplexes, cleaves the RNA strand, and releases intact DNA [22, 23]. The templating of an ASO to its target RNA mimics this RNA–DNA pairing, which prompts RNase H to cleave the RNA transcript while the intact ASO is released to bind new transcripts. Cleaved transcripts cannot be translated to protein, so repeated ASO binding events will exhaust any pools available for translation, resulting in sustained protein reduction. As described earlier, ASOs operating by this mechanism must have at least a portion containing bases unmodified at the 2' position, such as with a gapmer design.

Fully modified ASOs bind to a target transcript with high affinity and act through non-degrading mechanisms



**Fig. 2** Antisense oligonucleotide-mediated degradation of a gene target. Once delivered to the cerebrospinal fluid, antisense oligonucleotides (ASOs) are taken up into cells and can enter the nucleus. ASOs then bind to their gene target in a complementary fashion, creating a RNA–DNA hybrid that recruits the enzyme RNase H to cleave the target RNA. This action results in degradation of the gene target but spares the ASO, allowing it to persist in the cell. Without the RNA template, the gene will not be translated into protein, and the protein product is reduced over time. ASO therapeutics under investigation for amyotrophic lateral sclerosis (ALS) commonly employ this RNA-degrading strategy. Figure created with BioRender.com

by sterically hindering *trans*-acting elements at the targeted sequence. This property allows fully modified ASOs to influence mRNA processing steps, such as alternative splicing or translational control [10]. Splicing events occur at ~90% of mRNA transcripts in human cells, and each requires that a full complement of splice factors act in concert at stereotyped sequence motifs. Thus, ASOs that occlude recognition motifs for splice enhancers/repressors or that mask conventional intron/exon junctions can redirect mRNA splicing [24]. Non-degrading ASOs may also modify RNA translation, stability, or localization by targeting critical post-transcriptional regulatory hubs or *trans*-acting elements, such as microRNAs (miRNAs), that act on these hubs [25–27].

### Therapeutic Uses for ASOs and Current Limitations

Current challenges for some ASO therapies, particularly those with central nervous system (CNS) targets, include immune stimulation and CNS delivery. Toll-like receptors recognize foreign DNA and respond to specific sequence motifs [12, 28]. For example, CpG oligodeoxynucleotides activate toll-like receptor 9 to induce a potent interferon response [12, 28]. At high concentrations, early ASOs with a PS backbone could stimulate an inflammatory response, but advancements in backbone and 2' sugar modifications now can mitigate some undue immune stimulation [12, 13]. ASOs cannot readily cross the blood brain barrier. Thus, administration of ASOs to the CNS in preclinical and clinical testing requires intracerebroventricular (i.c.v.) and intrathecal routes, respectively, into the cerebrospinal fluid (CSF). Fortunately, direct injection into the CSF allows the ASO to distribute throughout the CNS, and initial trials using *SOD1*-targeted ASOs have demonstrated intrathecal administration as a well-tolerated, safe, and efficacious approach to ASO delivery [29–31]. New delivery approaches such as conjugating ASOs to lipid nanoparticles or cholesterol may soon enable peripheral administration or cell type targeting in the CNS [32–34].

Despite these challenges, the sequence and chemistry of ASOs make them powerful and precise preclinical investigational tools that can be designed with clinical translation in mind. ASOs that recruit RNase H are particularly useful in combating disease processes linked to toxicity of a single gene product (protein or RNA), a feature shared across many neurodegenerative diseases [10]. Non-degrading ASOs have also achieved remarkable success, most notably with the FDA approval of the splice-switching ASO nusinersen for the treatment of SMA [35, 36]. New uses for ASOs are under active investigation that may soon broaden their applicability to other disease mechanisms, such as genetic haploinsufficiency.

## ASOs Targeting *SOD1*

Mutations in *SOD1* account for about 2% of all ALS cases and 15–25% of familial cases [37]. Post-mortem tissue from patients with *SOD1*-ALS demonstrates *SOD1* aggregation, motor neuron degeneration, and muscle denervation. These features also appear in rodent model systems that express mutant human *SOD1* [38] but are absent in rodents lacking *SOD1* [39], supporting a toxic GOF mechanism for mutant *SOD1*. Transgenic mice and rats carrying copies of human mutated *SOD1*<sup>G93A</sup> are a well-validated cornerstone of preclinical ALS research. Like *SOD1*-ALS, *SOD1*<sup>G93A</sup> rodents develop aggregates of *SOD1* in the cytoplasm of motor neurons, exhibit progressive motor neuron loss and weakness, and die prematurely [40, 41]. Mutations in *SOD1* also exacerbate disease processes including oxidative stress [42], mitochondrial dysfunction [43], and excitotoxicity [44]. Collectively, these studies support the prediction that reducing *SOD1* might slow ALS progression.

### Preclinical

The first ASO therapy to target and decrease *SOD1* protein emerged from testing in human cells and mutant *SOD1* animal models [45]. The first candidate *SOD1*-lowering ASOs achieved robust, dose-dependent responses in cell culture, and i.c.v. infusion into a non-transgenic rat afforded > 60% knockdown of *SOD1* mRNA throughout the brain and spinal cord [45]. The widespread distribution of ASO throughout the brain and spinal cord as demonstrated in this first study was unexpected given that ASOs are relatively large molecules and highly charged [8]. This distribution remains incompletely understood but has been documented in multiple subsequent studies that are reviewed below. When tested in *SOD1*<sup>G93A</sup> rats, ASOs reduced *SOD1* mRNA by 40–60% throughout the brainstem and spinal cord and extended survival by 37% after disease onset [45]. As discussed below, these studies along with toxicology studies led to a successful phase 1 study (NCT01041222) using candidate ASO 333611, which was subsequently redeveloped to incorporate more advanced ASO technologies. Next generation ASOs containing 2'-MOE and 2'-O-ethyl modifications were advanced through a development pipeline, demonstrating superior target engagement and stronger knockdown over ASO 333611 in in vitro and in vivo pharmacokinetic studies [46]. In addition, i.c.v. injection of these ASOs into transgenic *SOD1*<sup>G93A</sup> rodents before disease onset profoundly extended survival, slowed motor impairment, and dampened neuromuscular injury [46]. ASOs also decreased serum levels of phosphorylated neurofilament

heavy chain (pNFH), which increases alongside neuronal injury and has emerged as an important biomarker for ALS [1]. When delivered after disease onset, ASOs suppressed further increases in neurofilament and remarkably restored neuromuscular activity to near baseline. When advanced to nonhuman primates (NHPs), intrathecally delivered ASOs achieved favorable target engagement and distribution [46]. ASOs lowered *SOD1* in NHPs across the CNS in a dose-dependent manner, with the highest dose achieving ~50% knockdown throughout the spinal cord. The profound functional rescue observed in rodent models and favorable pharmacokinetics in NHPs motivated another phase 1 a/b clinical trial (NCT02623699) for the new ASO BIIB067, discussed below.

Tracking ASO target engagement and patient response to therapy would enable real-time feedback of ASO efficacy and help inform upon trial design. To achieve this goal, a reasonable approach might be to measure the targeted protein from the CSF as a surrogate measure of ASO activity. However, *SOD1* has a long half-life in the CSF (>20 days) [47], so therapies that lower its expression would lag actual ASO activity. An alternate approach might be measuring changes in *SOD1* synthesis, rather than absolute levels. This strategy using stable isotope labeling and mass spectrometry was demonstrated in *SOD1* transgenic mice treated with *SOD1*-lowering ASO [48]. At 30 days post i.c.v. injection, *SOD1* in the CSF was minimally lowered, but a > 50% reduction in newly synthesized *SOD1* labeled with <sup>13</sup>C<sub>6</sub>-leucine could be detected as early as 10 days after dosing [48]. Importantly, levels of labeled *SOD1* matched *SOD1* mRNA levels observed in tissue from the brain and spinal cord. For human clinical trials, this approach could provide an early measure of target engagement in CNS tissues and guide trial design for CSF draws and dosing.

### Clinical

Despite common neuropathology, individuals with *SOD1*-ALS may display considerable heterogeneity in clinical presentation and history [49]. Of note, a natural history study on *SOD1*-ALS cases reported that the specific mutation in *SOD1* is an important modifier for disease progression rate [50]. For example, the *SOD1*<sup>A4V</sup> mutation comprises most *SOD1*-ALS cases in the USA and results in a characteristically aggressive disease. *SOD1*<sup>A4V</sup> and several other mutations are now associated with “fast-progressing” disease, which has become an important measure to stratify clinical trial group design. Incorporating these and other modifiers for disease will be crucial for designing and interpreting clinical trials that are powered to detect treatment effects.

Convincing target engagement and disease rescue from initial animal studies combined with an acceptable profile in toxicology studies prompted a phase 1, double-blind,

placebo-controlled clinical trial to study the safety and tolerability of intrathecally delivered ASO 333611 in *SOD1*-ALS patients [30]. This trial marked the first-in-man for a CNS-targeted ASO, predating trials of nusinersen or similar ASO. Given the first-in-man nature of the study, a single course (12 h slow intrathecal infusion) of a low dose (0.15 to 3 mg) was given. Results from this trial (NCT01041222) supported intrathecal infusion of ASOs as a favorable and well-tolerated route for administration to the CNS with no serious adverse effects. Although a reduction in SOD1 protein levels in the CSF was not detected, the trial tested conservatively low doses and a short time interval [30]. These studies [30, 47] were a landmark for ASO therapy, demonstrating for the first time that intrathecal infusion of ASOs is safe in humans and efficacious in animals and establishing a pipeline to rapidly advance ASOs from initial selection to clinical trials.

Following the initial SOD1 ASO trial, a phase 1–2, randomized, double-blind, placebo-controlled trial (VALOR; NCT02623699) was conducted starting in January 2016 to assess tolerability and pharmacokinetics of tofersen, the redeveloped *SOD1*-targeting ASO (BIIB067) in patients with *SOD1*-associated ALS [29]. Fifty participants were recruited and assigned to one of four dose cohorts (20, 40, 60, or 100 mg). Within a cohort, each participant was randomized to receive five doses of tofersen or placebo administered by five intrathecal injections over 12 weeks. Unlike the first *SOD1* ASO trial where the ASO was delivered via intrathecal catheter and external pump, this trial (and subsequent ASO trials) used an intrathecal bolus.

Tofersen was overall well-tolerated and safe. The most common adverse events were minor and related to lumbar puncture. Consistent with predicted SOD1 pharmacokinetics in CSF, SOD1 protein levels in the CSF were unchanged through study day 30. At study day 85, SOD1 was reduced in the 40 mg (27%), 60 mg (21%), and 100 mg (36%) groups, demonstrating effective target engagement [29]. Forty-two percent of participants receiving tofersen had lymphocytic pleocytosis, compared with 8% in placebo groups, although the significance of this finding is unclear.

Exploratory clinical measures in this trial included functional rating, lung vital capacity, muscle strength by handheld dynamometry, and both phosphorylated heavy chain (pNFH) and neurofilament light chain (NfL) concentrations in the CSF and plasma. Subgroup post hoc analysis for exploratory measures was performed for participants with fast-progressing disease. Though not powered to detect an effect on clinical measures, the progression of disability was tracked with ALS Functional Rating Scale-Revised (ALSFRS-R), which is a well-established, investigator-obtained scale [1, 51]. There was a trend toward slowing of decline in ALSFRS-R measured in the 100 mg group, which was further magnified in the fast-progressor subgroup. This

trend was also observed for predicted slow vital capacity and handheld dynamometry mega-score. Levels of plasma and CSF pNFH and NfL were reduced by day 85 in the 100 mg group compared to the day 1 baseline or placebo group. These promising results also supported segregating analysis on slow and fast progressors in subsequent trials.

The favorable safety profile and effective target engagement achieved in phases 1–2 motivated the advancement of tofersen to a 28-week phase 3, randomized, double-blind, placebo-controlled trial (VALOR; NCT02623699). This study evaluated the efficacy and safety of intrathecally administered tofersen at 100 mg (top dose from phases 1–2) in *SOD1*-ALS patients. A total of 108 participants with clinically manifest disease were enrolled and randomized to tofersen ( $n=72$ ) or placebo ( $n=36$ ) treatment arms. Consistent with analysis from the tofersen phase 1–2 trial [29] and to reduce the effects of the known heterogeneity of this genetic population, subgroups of fast and slow progressive patients were prespecified with the effect on progression in the fast-progressing group as the primary outcome. Sixty participants were enrolled for “fast-progressing” ALS. This trial continued through 2021, and results from this study were presented at the American Neurological Association Annual (ANA) Meeting in October 2021. A phase 3 long-term, open-label extension study (NCT03070119) continues to follow participants who received tofersen to assess long-term safety, tolerability, and efficacy.

Data slides from the ANA meeting were made publicly available and are the source of the results described here. The most common adverse events with tofersen at 100 mg were related to the lumbar puncture procedure [31]. Serious adverse events occurred at similar rates for tofersen (18.1%) and placebo (13.9%). No placebo-treated participants discontinued the study, compared with 5.6% of participants receiving tofersen. Of note, 4.8% of tofersen-receiving patients reported a severe neurologic event, including two cases of reversible transverse myelitis. There were increases in CSF protein in both placebo (8%) and tofersen (13%)-treated groups and a “shift to high leukocytes” in only tofersen-treated groups (10%). The significance of these CSF changes remains unclear. No data have yet been presented regarding the correlation of these changes with clinical outcomes or other measures, but this information will be important for understanding these data.

The study missed the primary outcome for efficacy, which was change from baseline across 28 weeks in ALSFRS-R [31]. However, multiple secondary and exploratory endpoints supported favorable clinical and biomarker trends for tofersen treatment. Within the fast-progressing group, tofersen decreased CSF SOD1 (38%) and plasma NfL (67%). Slow progressors showed similar but smaller reductions in SOD1 (26%) and NfL (48%). Among fast progressors, tofersen showed trends toward benefit in respiratory (slow vital

capacity) and muscle strength (handheld dynamometry) with differences between placebo and tofersen most apparent in the open-label extension (OLE). Slow progressors initially treated with tofersen showed very little decline in ALSFRS-R, slow vital capacity, and muscle strength, and slow progressors on placebo showed some apparent disease stabilization after being treated with tofersen in OLE. Data from this study are anticipated to continue to emerge and will be important to understand the long-term trends in OLE as well as the effect on survival.

Given the favorable safety profile and clear evidence of SOD1 protein lowering, the *SOD1* ASO is now being considered for treatment of asymptomatic gene carriers. A reasonable hypothesis is that treating individuals with *SOD1*-lowering drugs at the earliest evidence of biomarker changes, but prior to development of overt motor neuron disease, may slow or prevent their decline to clinically manifest disease. This prediction has motivated another phase 3 trial using tofersen (ATLAS; NCT04856982) to investigate its efficacy in presymptomatic adults with *SOD1* mutations who have elevated neurofilament indicating imminent phenocopy. This randomized, placebo-controlled trial will recruit ~150 participants and extend from May 2021 to August 2027. Presymptomatic participants without clinically manifest ALS will be followed longitudinally and will have frequent monitoring of serum NfL levels. If a participant exhibits high NfL, they will then be randomized to receive either 100 mg tofersen or placebo monthly for 2 years. An open-label extension period will allow participants who develop clinical ALS to receive tofersen in the unblinded arms of the study. The primary measure in this trial is the percentage of participants that clinically manifest ALS within 12 months of treatment. Important secondary measures include time to manifest ALS, ALSFRS-R scores, and concentrations of CSF SOD1 and plasma NfL.

## ASOs Targeting *C9orf72*

Hexanucleotide expansion in intron 1 of *C9orf72* is the most frequent genetic cause of frontotemporal dementia (FTD) and ALS, accounting for ~40% of fALS cases [4–6]. How *C9orf72* expansion causes disease remains an open question, but post-mortem samples and model systems of disease have highlighted multiple mechanisms contributing to *C9orf72* pathogenicity [52]. First, the mutant *C9orf72* gene can be transcribed bi-directionally, resulting in expression of sense and antisense RNA strands. Toxicity may occur from the intranuclear deposition of sense and antisense *C9orf72* into RNA foci and the folding of the GGG GCC repeats into a G-quadruplex, which itself can sequester important RNA-binding proteins and chromatin modifiers [53–55]. Second, both strands can produce strings of

dipeptide repeats (DPRs), owing to a noncanonical repeat-associated non-ATG-mediated (RAN) translation mechanism. These dipeptides may be toxic, perhaps by disrupting proteostasis or nucleolar machinery [56–59]. Intracellular deposits of five DPRs appear in human post-mortem tissue, further supporting a toxic GOF mechanism [60–62]. Third, haploinsufficiency of *C9orf72* limits its physiological functions, which include vesicle trafficking, autophagy, lysosomal processing, and immune response [63, 64], suggesting loss of normal *C9orf72* can contribute to disease [65]. It is likely that both gain- and loss-of-function mechanisms synergize to cause disease [52, 66]. Altogether, the above findings largely support ASO reduction of expanded *C9orf72* RNA, but not wild-type *C9orf72*, as a promising therapeutic strategy to reduce toxicity due to DPRs and repeat-containing RNA.

## Preclinical

Cells derived from patients with *C9orf72* expansions phenocopy key cellular features seen in post-mortem tissue, including intranuclear RNA foci and intracytoplasmic DPR deposits [67]. Because these features are likely toxic and an immediate consequence of mutant *C9orf72* expression, these provide excellent read-outs for ASO efficacy in suppressing *C9orf72* and resolving toxicity. Three reports, published almost simultaneously in 2013, were first to explore the therapeutic potential of ASO-mediated degradation of *C9orf72* [68–70]. When delivered to patient-derived fibroblasts [68], iPSC neurons [70], or motor neurons [69, 70], *C9orf72*-targeting gapmer ASOs could potentially reduce repeat-containing *C9orf72* transcripts and clear intranuclear RNA foci. Since 2013, new ASO formulations have shown promise in cellular models. Two studies further demonstrated that ASOs with a stereopure backbone [71, 72] or mixed backbone containing LNAs [73] also could reduce expansion-containing transcripts and attenuate pathology using *C9orf72*-ALS patient-derived cells.

One unique opportunity for *C9orf72* targeting comes from the multiple transcript variants produced. Splice variants 1 and 3 contain the intron that harbors the expansion, whereas variant 2 does not. Variant 1 leads to a short isoform protein; variants 2 and 3 produce the same long isoform proteins. By targeting intron 1, multiple groups [68–73] (referenced above) have achieved lowering of the expansion-containing transcripts while preserving variant 2 and thus not further suppressing *C9orf72* protein levels. This quality is important because it is likely not desirable to degrade all *C9orf72* transcripts, as *C9orf72* protein has important physiological functions [52]. However, one remaining challenge for *C9orf72* is that the expanded *C9orf72* DNA is transcribed bi-directionally, producing both sense and antisense RNA strands that deposit in RNA foci. At present,

all ASOs in development only bind and degrade the sense strand, while DPRs and RNA foci produced from antisense *C9orf72* remain. In cellular studies, *C9orf72*-targeting ASOs did decrease RNA foci, but this reduction occurred predominantly through clearance of foci composed of sense strand RNA without affecting antisense foci [68]. This inability to neutralize simultaneously both sense and antisense strands remains an important limitation to current *C9orf72* ASO therapies [70].

Transgenic mice carrying a bacterial artificial chromosome with the full human repeat-containing *C9orf72* gene are commonly used rodent models for *C9orf72*-associated disease [74, 75]. These animals develop RNA foci and DPR proteins throughout the cortex and spinal cord but do not exhibit neurodegeneration or motor impairments. Some, however, develop progressive cognitive impairment and anxiety phenotypes reminiscent of FTD [74]. Across multiple studies [71–74], ASOs targeting *C9orf72* were delivered by i.c.v. injection prior to phenotypic onset. ASOs decreased repeat-containing *C9orf72* throughout the brain and spinal cord without affecting total *C9orf72* RNA. Expectedly, ASOs decreased DPR levels and frequency of sense foci by > 50%, without appreciable effects on amounts of antisense foci [72–74]. Intron-targeting ASOs delivered after phenotypic onset lowered DPRs [72–74] and rescued behavioral assays for anxiety and cognitive function to near levels seen in non-transgenic mice at 12 or 15 months [74]. These encouraging data from cellular and animal models have since motivated testing in humans. Below, we discuss a phase 1 trial (NCT03626012) for the ASO BIIB078, a recently initiated phase 1b/2a trial (NCT04931862) for the stereopure ASO WVE-004 [71, 72], and early results from a single individual receiving the mixed backbone ASO afinersen under an Investigational New Drug (IND, “compassionate use”) application [73].

With clinical trial design in mind, measuring RAN proteins produced from expanded *C9orf72* RNA could provide a way to track ASO target engagement and monitor response to therapy [76]. The RAN product poly(GP) is produced from RAN translation of both *C9orf72* RNA strands and can be detected in the CSF of mutation carriers. Notably, CSF poly(GP) is present only in *C9orf72* individuals and remains stable over time in expansion carriers despite disease progression [76, 77]. Upon knockdown of *C9orf72* by ASOs in repeat-expressing transgenic mice, poly(GP) in the CSF sharply falls, which correlates robustly with reductions observed in tissue poly(GP) levels, RNA foci, and repeat-containing *C9orf72* RNA. Altogether, the specificity of poly(GP) to mutation carriers, the stability across disease progression, and its strong correlation to cellular pathology post-treatment make CSF poly(GP) an excellent pharmacodynamic marker that could be used in emerging clinical trials [76].

## Clinical

Understanding clinical measures in *C9orf72*-ALS is critical for designing powered studies that target *C9orf72*-ALS patients and measure clinical outcomes most relevant to this population. A natural history study tracking *C9orf72*-ALS disease identified that *C9orf72* cases have more homogenous clinical presentation, later onset, and largely similar disease duration, compared to sporadic cases [78]. As a biomarker, CSF levels of poly(GP) are stable over disease course and can segregate *C9orf72*-ALS from sporadic cases. Importantly, CSF poly(GP) correlates strongly with poly(GP) concentrations in post-mortem cortex and cerebellum [78]. These findings should provide guidance in designing appropriately powered studies and support measuring poly(GP) in the CSF as a correlate to target engagement.

Based on favorable results using ASOs in patient-derived cells [68–70] and transgenic mice [74], a randomized, placebo-controlled phase 1 trial (NCT03626012) was conducted to examine the safety and tolerability of the *C9orf72*-lowering ASO BIIB078. In this first-in-man, ascending dose trial, 114 participants with *C9orf72*-ALS were recruited and administered BIIB078 or placebo by intrathecal infusion. Inclusion criteria also required a prestudy ALSFRS-R slope of less than 0.4 points per month, which excludes “fast progressors.” This trial continued from September 2018 to November 2021, and early results were announced in a press release in March 2022 [79]. BIIB078 was well-tolerated with only mild to moderate adverse effects. Secondary endpoints evaluated pharmacokinetics and clinical measures, such as change in ALSFRS-R, slow vital capacity, and muscle strength. None of these endpoints were met, and participants that received BIIB078 trended toward greater clinical decline. Further development on BIIB078 has been discontinued.

A randomized, placebo-controlled phase 1b/2a trial (NCT04931862) was also initiated for the stereopure *C9orf72*-lowering ASO WVE-004 [71, 72] in patients with ALS and/or FTD due to *C9orf72* expansions. WVE-004 or placebo will be administered intrathecally to participants at four ascending doses to evaluate safety and tolerability. Secondary outcome measures will assess pharmacokinetics and pharmacodynamics, including changes in poly(GP) levels in the CSF. This trial began in July 2021 and is expected to continue through February 2023 with estimated enrollment of 42 participants. A press release in April 2022 announced early results for a portion of participants. Most adverse events related to WVE-004 were mild to moderate, and no elevations in CSF leukocytes or proteins were observed. A single dose of WVE-004 achieved durable reductions in poly(GP) in the CSF, indicating successful target engagement. No change in clinical outcome

measures was observed in participants receiving WVE-004, and CSF NfL was slightly elevated.

An IND application (IND141673) was approved by the FDA for the testing of the mixed backbone ASO afinersen in a single participant, a 60-year-old male with a *C9orf72* expansion [73]. Prior to this application, toxicology assessments in rodents, sheep, and monkeys supported safety of afinersen [73]. At treatment initiation, this individual had mild motor changes with elevated poly(GP) in the CSF. Eight escalating doses were administered intrathecally over 60 weeks, starting in August 2019. Afinersen distributed well throughout the CSF, and compared to baseline, CSF poly(GP) levels dropped by 80%, suggesting that afinersen engaged and decreased *C9orf72*. ALSFRS-R remained stable throughout treatment. In contrast with studies of tofersen for *SOD1*-ALS, afinersen treatment was associated with increased pNFH and NfL in the CSF [73]. At the time of this review, no clinical trial is yet registered for afinersen.

## ASOs Targeting *FUS*

Mutations in fused in sarcoma (*FUS*) cause a rare and aggressive form of ALS with early and often juvenile onset [7]. *FUS* is an RNA-binding protein that is critical in DNA repair and RNA metabolism, including the splicing and translation of mRNA [80]. ALS-associated mutations in *FUS* may cause partial loss of function (LOF), but mechanistic studies have demonstrated GOF mechanisms are significant drivers of motor neuron loss in *FUS*-ALS [81]. While most forms of ALS commonly show TDP-43 pathology, postmortem tissue from *FUS*-ALS patients exhibits intracytoplasmic aggregation of *FUS* in the absence of TDP-43 pathology [82]. The role of *FUS* in RNA metabolism requires frequent translocation between the nucleus and cytoplasm [80], but mis-localization and sequestration of *FUS* in aggregates likely impairs normal functions, which argues for a LOF mechanism. However, this conclusion contrasts with findings from in vivo models in which loss of *FUS* in motor neurons induced no motor neuron loss or denervation [83], whereas transgenic mice overexpressing wild-type *FUS* develop aggressive motor neuron loss and intracellular inclusions composed of *FUS* and other RNA-binding proteins [84]. Furthermore, intracellular aggregates of *FUS* appear to sequester or decrease solubility of other RNA-binding proteins, such as hnRNPA1 or UPF1, that are critical for RNA metabolism. Collectively, these studies using *FUS*-ALS human tissue and rodent model systems support a toxic GOF mechanism contributing to *FUS*-ALS. Therefore, ASO therapies that decrease *FUS* expression may hold promise for individuals with *FUS*-ALS.

## Preclinical

Knock-in mice that express wild-type and mutant *FUS*<sup>P525L</sup> from the *FUS* locus develop progressive loss of spinal motor neurons and intracellular aggregates of insoluble *FUS* but do not exhibit motor impairments or reduced survival [85]. Identified from in vitro screen, the ASO ION363 targeting the 6th intron of *FUS* decreases expression of both mutant and wild-type transcripts. I.c.v injection of ION363 in *FUS*<sup>P525L</sup> mice decreased both mutant and wild-type *FUS* by 20–50% in the brain and spinal cord by 1 month post-injection. ION363 reduced levels of insoluble *FUS* and insoluble RNA-binding proteins that associate with *FUS* aggregates, such as hnRNPA1. Moreover, ION363 prevented neurodegeneration of lumbar motor neurons and loss of neuromuscular junction innervation, which were sustained at 4 and 6 months [85]. Reversal of neurodegeneration and reductions in *FUS* in *FUS*<sup>P525L</sup> mice motivated an IND (“compassionate use”) application for testing in humans with *FUS* mutations [85].

## Clinical

An IND application was approved by the FDA for the use of ION363 in a single ALS patient with a *FUS*<sup>P525L</sup> mutation (age 26). Under this application, no toxicology assessments in rodents or monkeys were needed. The participant received 12 injections between June 2019 and March 2020 before her dying from ALS in May 2020 [85]. She received monthly ascending doses intrathecally, starting from 20 mg up to maximum 120 mg. Treatment began at 6 months post-clinical onset, at which point her disease reached advanced stage. The participant had no serious adverse events. Since her death, more than a dozen individuals carrying *FUS* mutations with presymptomatic or symptomatic ALS have also received ION363 through Expanded Access Program IND applications.

Neuropathological examination was performed for the first participant, alongside a non-ALS control and an ALS patient with the *FUS*<sup>P525L</sup> mutation who did not receive therapy [85]. Relative to ALS control, the *FUS* ASO-treated participant had less total *FUS* and mutant *FUS* protein, including insoluble *FUS*, in the lumbar spinal cord. Levels of other insoluble RNA-binding proteins, such as hnRNPA1, were also lower. In the spinal cord and motor cortex, there was little nuclear *FUS* staining, and aggregates containing *FUS* in motor neurons were sparse compared to the untreated ALS-*FUS*<sup>P525L</sup> control in which *FUS* aggregates were abundant. These examinations suggest that ION363 treatment in this participant correlates with reduced *FUS* pathology.

A phase 3 clinical trial (FUSION; NCT04768972) to assess efficacy, safety, and pharmacology of ION363 (now jacifusen) was initiated for patients with *FUS*-ALS. Over



April 2021 through March 2024, 64 patients will be enrolled and assigned to jacifusen or placebo at a 2:1 ratio, including any who had previously received jacifusen under IND applications. Jacifusen will be administered monthly or bimonthly for 29 weeks in part 1 of the study. Part 2 of the study will be a subsequent 72 week open-label extension period in which all participants will receive jacifusen. A unique component of this trial is the involvement of a “rescue.” Specifically, if a participant shows significant functional decline during part 1, they will be moved to the part 2/open-label extension of the study. The primary outcome is a joint rank analysis of ALSFRS-R, time to rescue (as described above), and ventilation assistance-free survival. Secondary outcomes include muscle and lung function, survival, and change in CSF FUS protein and neurofilaments.

## ASOs Targeting *ATXN2*

Trinucleotide repeat expansions in ataxin-2 (*ATXN2*) are a cause of the neurodegenerative disease spinocerebellar ataxia type-2 (SCA2) and an identified risk factor for ALS. *ATXN2* is an important regulator of mRNA polyadenylation and stress granule assembly that contains an unstable polyglutamine (polyQ) tract encoded by repeats of CAG [86]. Normal alleles usually have 22 or 23 repeats, while patients with SCA2 carry an allele with a longer expansion of >34 repeats [87]. Intermediate length expansions of 27–33 repeats in *ATXN2* are now recognized as a significant heritable modifier of ALS risk [7]. The link between *ATXN2* and ALS was first identified when a genetic screen in yeast found that upregulating Pbp1 (*ATXN2* homolog) severely worsens cell survival due to TDP-43 overexpression, while lowering Pbp1 suppresses this toxicity [88]. This finding was important, as the nuclear depletion, cytoplasmic mislocalization, and pathologic aggregation of TDP-43 are hallmarks across > 95% of ALS cases. Furthermore, mutations in the gene encoding TDP-43, *TARDBP*, are a well-established cause of familial ALS. Thus, TDP-43 dysfunction likely plays a critical role in the vast majority of ALS cases and targeting *ATXN2* may limit TDP-43 dysfunction. Neuropathological examination of post-mortem tissue from patients with ALS with and without intermediate length *ATXN2* expansions also revealed that spinal motor neurons often contain large intracytoplasmic deposits of *ATXN2* [88, 89]. In human population genetics studies, sequencing for polyQ repeat lengths from >900 patients with ALS and control cases revealed a 2.8-fold enrichment of intermediate expansions in the ALS cohort, detected in 4.8% of cases [88]. These results have since been validated in additional population-based cohorts, for example, showing 5.4- [90], 4.8- [91], or 3.0-fold [92] enrichments.

Mounting evidence supports *ATXN2* and intermediate-length polyQ expansions in *ATXN2* in altering RNA metabolism or stimulating the pathological transformation and deposition of TDP-43. First, intermediate length expansions can directly promote TDP-43 aggregation by activating caspases and downstream effectors that cleave and phosphorylate TDP-43, though these events do not occur with non-expanded *ATXN2* [93]. Second, intermediate length expansions increase the stability of *ATXN2* and allow for greater molecular association with TDP-43, which is especially apparent under stress conditions when stress granules are induced [88]. While other mechanisms related to RNA processing or receptor trafficking may be involved, it is proposed that non-expanded *ATXN2* modifies disease through modulating stress granule dynamics. Stress granules are dynamic, membrane-less cytoplasmic condensates composed of RNA and proteins that assemble during cellular stress to temporarily stall translation [94]. If cellular stress does not resolve, such as in disease, stress granules persist, which may limit protein production, sequester critical proteins, and provide a crucible for RNA-binding proteins to interact [95]. As *ATXN2* is a critical organizer of stress granules, the increased stability afforded to intermediate length expansions in *ATXN2* might exacerbate disease by affecting their resolution or prolonging sequestration [86]. For example, TDP-43 often localizes within stress granules, and these condensates may promote TDP-43 self-association and mis-localization to the cytoplasm [88, 96]. As alluded to above, *ATXN2* modifies cell toxicity or survival due to overexpressed TDP-43 in yeast [88], flies [88], and mice [97]. Because TDP-43-proteinopathy and disrupted RNA processing are hallmarks across most forms of ALS [98], the ability for *ATXN2* to modify these processes makes *ATXN2* an attractive target beyond *ATXN2* expansion-related ALS.

## Preclinical

ASO development and preclinical testing for *ATXN2* knock-down were simultaneously performed for model systems of *ATXN2*-repeat expansion disease SCA2 and an ALS model system of TDP-43-proteinopathy [97, 99]. In the initial development of ASOs for SCA2, a promising ASO candidate was selected that targets exon 11 of human *ATXN2* [99]. In two SCA2 models of *ATXN2* expansion (*ATXN2*-Q127 and BAC-Q72), ASOs reduced human *ATXN2* ~ 75% in the cerebellum, cortex, and spinal cord. This result was accompanied by striking rescue of disease-relevant gene expression and functional readouts, such as firing frequency of cerebellar Purkinje cells [99]. This study was a promising demonstration of *ATXN2*-lowering therapy in a model of *ATXN2*-expansion disease, supporting its translation to animal models of ALS.

*ATXN2* lowering therapy for ALS was evaluated in a transgenic mouse model of TDP-43-proteinopathy that drives pan-neuronal expression of human wild-type TDP-43 [97]. This model is aggressive and results in rapid and robust motor dysfunction and death around postnatal day 24. Genetic ablation of *ATXN2* dramatically extended lifespan by 80%, limited gait dysfunction, and slowed disease progression [97]. Consistent with a stress granule-mediated mechanism, loss of *ATXN2* did not affect total TDP-43 levels but did reduce TDP-43 inclusions. When administered on postnatal day 1 to TDP-43-proteinopathy mice, an *ATXN2*-lowering ASO extended lifespan by 35% and improved gait performance, despite the aggressive decline in this model system [97]. These studies supported the initiation of a clinical trial for *ATXN2*-lowering ASOs in *ATXN2*-associated ALS and sporadic ALS.

## Clinical

A randomized, placebo-controlled phase 1 trial (NCT04494256) was initiated in September 2020 to evaluate the safety and pharmacokinetics of an intrathecally delivered ASO designed to lower *ATXN2* (BIIB105) in patients with *ATXN2*-associated ALS and sporadic ALS. The study expects enrollment of 70 participants with an end date of February 2023. Dosing arms will segregate individuals with sporadic ALS from *ATXN2*-associated ALS. Within each group, participants will be assigned to receive placebo or one of five sequentially escalating doses of the *ATXN2* ASO.

## Limitations and Future Directions

### Limitations to Treating ALS with ASO Therapy

Limitations to treating ALS with ASOs fall into three general categories: challenges associated with the disease itself, ASO pharmacokinetic/pharmacodynamic properties, and potential on and off target toxicities of the ASO. Regarding ALS disease, there may be challenges in initiating any therapy after the disease has started. While there are good examples of reversals of function or pathology in animal models of neurodegeneration, it is not clear yet whether the neurodegenerative disease process may be halted in humans. Regarding ASO pharmacokinetic/pharmacodynamics, there are multiple favorable properties of ASOs including widespread distribution, long half-life, and proven target reduction in humans for multiple targets including *SOD1*. However, the details of drug delivery are not entirely clear in humans with limited data on correlations between CSF measurements and tissue levels. Whether an individual ASO has lowered the target sufficiently in the cell types of interest, for example, motor

neurons, remains incompletely understood. Toxicities may also limit the effectiveness of the ASO. On-target toxicity associated with lowering levels of the target gene product may introduce some unknown side effects. While the toxicity of a dominantly inherited mutation likely outweighs the risk of lowering the gene product, this possibility will need further consideration. Off-target toxicity, particularly activation of the immune system, has been a longstanding potential concern with ASO use. The presence of immune cells, elevated protein composition, and a few cases of myelitis seen in human clinical trials may indicate some pro-inflammatory effects. While the full data from the recent *SOD1* ASO trial are not yet available, there were no correlations observed yet between changes in CSF parameters and disease course or response to the ASO.

## New Targets for ASO Therapy

Dozens of genes are now linked with ALS, and each represents a putative target for ASO therapy. Most cases of fALS can be linked to mutations in *SOD1*, *C9orf72*, *FUS*, or *ATXN2*, which has likely hastened ASO development for these genetic forms of disease. ASOs are now in development to target genes associated with other forms of fALS. These may be supported by new ASO strategies that are under active investigation, including approaches to increase gene expression. ASOs that block the action of translational repressors on a target transcript might increase protein translation. Gene upregulation would have implications for treating haploinsufficiency disorders, such as *TBKI*-associated ALS, and for targeting pathways associated with sporadic ALS.

ASOs may also be effective therapeutics for non-genetic forms of ALS, as may be the case for *ATXN2*. *Stathmin-2* and *UNC13A* are two important splice targets of TDP-43 that are essential for neuron health and activity. Loss of nuclear TDP-43, a hallmark across ALS cases, induces mRNA splicing defects, and mis-splicing of *stathmin-2* and *UNC13A* causes their reduced protein expression and function [100–104]. ASOs that correct splicing defects and restore *stathmin-2* or *UNC13A* protein might ameliorate some downstream consequences of TDP-43-proteinopathy.

## Conclusions Regarding ASO as Therapeutics

The successful progression of ASOs through the drug development pipeline has made them strong candidates for many diseases with single gene mutations or prominent toxic species. Beyond ALS, ASO trials have consistently demonstrated effective target engagement and action, with a marked change to biomarkers that reflect disease processes.

The number of implicated genes for ALS grows with each year, and with the emergence of streamlined pipelines to clinical trial, ASO development for rare diseases becomes increasingly feasible. While complete understanding of ASO distribution and toxicities have lagged behind their clinical application, advancements in ASO technology will continue to expand their delivery, cell targeting properties, applicability in disease, and efficacy, enabling new promising therapeutics to keep pace with breakthroughs in ALS pathophysiology.

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