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SOD1-targeting therapies for neurodegenerative diseases: a review of current findings and future potential

Abstract

INTRODUCTION:

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with limited effective treatments. Mutations in the SOD1 gene are causative in approximately 2% of ALS cases. As the first ALS-associated gene to be discovered, efforts in the development of therapies targeting SOD1 are advanced relative to other genetic causes of ALS. Two SOD1-targeting strategies: antisense oligonucleotides and microRNA, have been trialled in humans to date, with preliminary evidence of disease-modifying activity.

AREAS COVERED:

In this review, the following areas are discussed: 1) the pathophysiology of mutant SOD1-ALS, and the rationale for targeting the SOD1 gene; 2) the strategies that have been used to target mutant SOD1 in clinical and preclinical studies; 3) the role of misfolded wild-type SOD1 in sporadic ALS and other neurodegenerative diseases, and the potential for targeting SOD1 in these patients; 4) future avenues for research. A literature search of publications pertaining to SOD1-ALS and its treatment from 1992-present using the MEDLINE database form the basis for this review.

EXPERT OPINION:

Central nervous system SOD1 knockdown is achievable in SOD1-ALS patients with intrathecal antisense oligonucleotide therapy, and is both safe and well-tolerated: phase III study outcomes are awaited. Well-designed virus-based delivery strategies for RNA interference therapies targeting SOD1 show promise in animal models and may, with caution, provide an effective treatment strategy if these results can be recreated in future clinical studies.

KEYWORDS: antisense, oligonucleotide, RNAi, familial ALS, tofersen, immunotherapy, superoxide dismutase, gene therapy, Parkinson's

1. SOD1 pathophysiology

1.1. Mutant SOD1 as a cause of familial ALS

Amyotrophic lateral sclerosis (ALS) is a devastating adult-onset neurodegenerative disorder characterised by progressive loss of upper and lower motor neurons. Clinically, this manifests as atrophy and spasticity of skeletal muscle, leading to paralysis. Dysphagia and dysarthria may also occur when bulbar muscles are affected. The prognosis is invariably fatal: survival time from symptom onset to death from respiratory weakness is typically between 2 and 4 years [1]. There is no cure currently available: one medication, Riluzole, has been used for many years and has been shown to modestly prolong life [2]. Another drug, Edaravone has since been licensed and may prolong functional independence in a subgroup of patients [3,4]. Recently, early phase clinical trials of two gene silencing therapies targeting SOD1 have been published with some preliminary evidence of disease-modifying activity in patients with ALS caused by mutations in the SOD1 gene [5,6]. It is hoped that these breakthroughs may herald a new era of personalised medicine for monogenic causes of ALS and other neurodegenerative diseases [7].

Approximately 10% of total ALS cases are familial (fALS) and usually display autosomal dominant inheritance: over 50 ALS-associated genes have been identified to date, but in approximately 30 percent of familial cases a specific genetic cause cannot be identified [8]. Mutations in the SOD1 gene are associated with 15-30% of all familial ALS cases, with a higher prevalence in Asian populations than in people of European descent [8]. Other significant monogenic causes of fALS include intronic repeat expansions of *C9orf72*, and missense mutations in *TARDBP*, and *FUS* [1,8]. Notably, repeat expansions of *C9orf72,* identified in 2011, represent the commonest known genetic cause of ALS in Europeans, accounting for a third of familial cases in these populations, and are also the commonest genetic cause of frontotemporal dementia (FTD) . The remaining 90% of total ALS cases are sporadic (sALS), but twin studies suggest a degree of heritability in ALS remains unaccounted for, and one or more genetic risk factors may be present

even in sporadic cases [9,10]. Indeed, mutations in SOD1 can be identified in 1.2-1.5% of sALS cases, and may represent *de novo* mutation or incomplete penetrance [8,11,12]. It is anticipated that genetic screening in ALS will become increasingly common clinical practice as therapies directed toward specific mutant genes are developed. The search for new ALS genes is ongoing.

Located on chromosome 21q22.11, the *SOD1* gene encodes cytoplasmic copper/zinc binding superoxide dismutase type 1 (SOD1). The SOD1 protein forms a homodimer with copper and zinc residues to form a metalloenzyme complex, which localises to the cytoplasm, nucleus and intermembrane space of mitochondria [13,14]. SOD1 is found abundantly in almost all cell types; it is estimated to make up approximately 1% of total soluble protein in the central nervous system [15]. Physiologically, the function of SOD1 is to catalyse the removal of superoxide ions ($O_2^{\cdot-}$) through dismutation to hydrogen peroxide (H_2O_2) and oxygen. $O_2^{\cdot-}$ ions are commonly generated as by-products of aerobic respiration in cells, and are a precursor to the formation of other reactive oxygen species (ROS) [14]. While ROS have important roles in cellular homeostasis, signalling and immune function, the imbalance of ROS production and elimination can result in damage to cellular structures as oxidative stress. The conversion of $O_2^{\cdot-}$ to H_2O_2 helps to relieve oxidative stress, as H_2O_2 is less reactive and can be further reduced to water [16].

After twenty-seven years of study, the pathophysiology of mutant SOD1-associated ALS has been better characterised than other forms of the disease. The $SOD1^{G93A}$ mouse was the first animal model to be generated for ALS, and remains in widespread use today [17,18]. The study of $SOD1^{G93A}$ mice, and other SOD1-based ALS models such as the slower-progressing $SOD1^{G37R}$ mouse, underpin a great deal of our current understanding of ALS pathophysiology in general. The precise cascade of events that drives neurodegeneration in mutant SOD1 ALS remains incompletely understood. Two competing theories evolved: 1) a toxic gain-of-function of the mutant SOD1 protein, and 2) toxic loss of dismutase function through haploinsufficiency. Subsequent

experimental evidence favoured the former, but controversies remain, as both mechanisms may play distinct roles in pathogenesis [19,20].

1.2.Toxic gain-of-function: the case for SOD1 silencing in SOD1-associated ALS

Clinical and experimental data point strongly toward a gain-of-function of mutant SOD1 as the predominant mechanism in mutant SOD1-associated ALS. This has made it an attractive therapeutic target using both genetic therapy approaches, and more conventional approaches such as small molecules and antibodies [17]. Over 200 ALS-associated mutations in the SOD1 gene have been described to date: the vast majority display autosomal dominant inheritance and are missense variants [17,21] [(http://alsod.iop.kcl.ac.uk/]. Definite pathogenicity has been confirmed in only a handful of these variants. In many of the more common ALS-causing SOD1 mutations (for example $SOD1^{A4V}$), normal or near-normal enzyme activity is observed. Others show reduced activity, but this does not correlate with the severity of the phenotype observed [22,23].

In $SOD1^{G93A}$ mice, disease severity is associated with higher copy numbers of the mutant SOD1 transgene, but is unaffected by wild-type (WT) SOD1 activity [24]. In rare human cases in which two dominant mutant genes have been inherited, disease onset is earlier, and the phenotype more aggressive [25,26]. Conversely, in cases with a co-inherited mutation in the SOD1 promoter leading to reduced mutant protein expression, disease onset is later [27]. Taken together, these findings imply a dose-dependent relationship between mutant SOD1 protein levels and toxicity.

1.3.Toxic gain-of-function: misfolding and aggregation

The exact properties of the mutant SOD1 protein that confer toxicity remain unclear, as ALS-causing mutations have been described along virtually the entire length of the protein and do not appear to show any specificity for the substrate binding site. Mutant SOD1 is known to misfold and form cytoplasmic aggregates (hyaline conglomerate inclusions) in patients and transgenic mice: they are a pathological hallmark of mutant SOD1-associated ALS. SOD1 misfolding *in vitro*

occurs most readily in the presence of mutations that promote oxidation, monomerization and demetallation of the protein [28,29]. Specifically, oxidation of a highly reactive free cysteine residue, Cys111, has been shown to delay maturation of the protein and promote toxic misfolding [30].

SOD1 aggregates are found throughout the motor cortex and spinal cord in patients and transgenic mice, and can be detected using conformation-specific antibodies. Aggregates are selectively toxic to cultured motor neurons *in vitro*, but are readily taken up by all cell types [31]. Mutant SOD1 aggregates can be extracted and purified from the CNS tissue of SOD1 rodent models exhibiting an ALS-like phenotype. When these purified SOD1 aggregates are inoculated into healthy SOD1 transgenic mice, they have been shown to propagate through the CNS in a prion-like manner and cause a premature ALS-like phenotype [32]. The precise role of these aggregates *in vivo* remains contentious however, as some rodent studies have shown that motor symptoms precede the accumulation of detectable SOD1 aggregates, implying that they may be a downstream effect of another pathogenic process rather than the cause [33]. Furthermore, a recent study showed that the accumulation of SOD1 aggregates actually predicted a less aggressive disease course in transgenic mice, but the opposite was true for misfolded, soluble, SOD1 [34]. This implies that sequestering of misfolded SOD1 in aggregates is actually a protective mechanism adopted by the cell.

1.4.Toxic gain-of-function: downstream effects of mutant SOD1

Mutant SOD1 has been implicated in disruption of an array of intracellular processes including: mitochondrial function, axonal transport, non-cell autonomous toxicity and regulation of membrane excitability (**Figure 1**).

Impaired mitochondrial function is thought to play a pivotal role in the pathogenesis of many neurodegenerative diseases [reviewed in 36]. Misfolded mutant SOD1 selectively associates with mitochondria in the intermembrane space, disrupting cell signalling, energy homeostasis and

axonal transport [36]. Interference in the electron transport chain by the mutant SOD1 protein also leads to excessive production of damaging ROS [37,38]. Mechanistically, mutant SOD1 may interact directly with a voltage-dependent anion channel (VDAC1), causing inhibition and preventing transport of adenosine diphosphate (ADP) across the outer mitochondrial membrane [39]. Bcl-2, a mitochondrial anti-apoptotic signalling protein, is bound by WT SOD1, but has been found to form toxic complexes when bound to mutant SOD1 through conformational change in bcl-2 and exposure of the toxic BH3 domain, leading to pro-apoptotic signalling [40,41]. Mutant SOD1 also directly interferes with axonal transport of mitochondria and mitophagy by upregulating degradation of outer mitochondrial membrane protein mitochondrial Rho GTPase 1(Miro)1 [42,43].

The accumulation of misfolded mutant SOD1 within the endoplasmic reticulum (ER) leading to ER stress is another putative toxic mechanism. Mutant SOD1 interacts with the ER protein derlin-1: this is thought to lead to cell death via activation of the apoptosis signal-regulating kinase (ASK1)-dependent cell death pathway [44].

Glutamate-mediated hyperexcitability leading to Ca^{2+}-mediated excitotoxicity is predicted to play a key role in pathogenesis of sALS and fALS, as well as other neurodegenerative diseases. Cerebrospinal fluid (CSF) glutamate levels are higher in ALS patients than those observed in healthy controls [45]. Electrophysiological studies in patients have revealed evidence of hyperexcitability in both sALS and SOD1-associated fALS, likely responsible for the clinical manifestations of muscle fasciculation and cramps [46,47]. Motor cortex hyperexcitability is observed in even in presymptomatic SOD1 mutation carriers who subsequently develop ALS and therefore likely represents an early feature of disease [48]. Mutant SOD1 has been shown to interact with the voltage-gated sodium channel NaV1.3 in a mouse embryo model, causing abnormal inward sodium currents which predispose to membrane hyperexcitability [49]. Furthermore, the mutant SOD1 protein has also been demonstrated to interfere with the reuptake of

the excitatory neurotransmitter glutamate in neurons and glia, further predisposing motor neurons to excitotoxicity [50,51].

Outside the neuron, non-cell autotoxicity mediated by glial cells is known to play a pivotal role in the motor neuron degeneration associated with both SOD1-associated fALS and sALS. It has been demonstrated that astrocytes and oligodendrocytes derived from ALS patients, including those carrying SOD1 mutations, are selectively toxic to healthy motor neurons when cultured together *in vitro*, through release of soluble factors and via direct cell contact [52,53]. Interestingly, the motor neuron toxicity afforded by oligodendrocytes derived from both sALS and SOD1-ALS patients occurs in a SOD1-dependent mechanism (but not in C9orf72-ALS-derived oligodendrocytes), and can be ameliorated with SOD1 silencing using RNA interference (RNAi, Figure 1) [52]. In transgenic mice, selectively removing the SOD1 transgene from microglia, oligodendrocytes, or astrocytes (as well as neurons) can delay the onset or progression of the ALS-like phenotype [54–56].

1.5. Toxic loss-of-function: reasons to exercise caution in SOD1 silencing

Since the discovery of the SOD1 association with ALS, oxidative stress has been postulated as a key player in disease pathogenesis [16,18,57]. Brain, spinal cord, CSF and blood specimens from sALS and fALS patients show biochemical markers of increased oxidative stress [58–61]. Preclinical studies of antioxidants as a potential therapy have showed promising results, and the licensed drug Edaravone may exert its mechanism of action as an antioxidant (Figure 1) [3,4]. However, several SOD1 knockout studies in the 1990s failed to demonstrate an ALS-like phenotype in mice [62,63]. These SOD1 knockout mice models continued to be used to simulate chronic oxidative stress: subsequent studies in these animals demonstrated age-related muscle atrophy, distal motor axonopathy and a modest reduction in lifespan (without evidence of neurodegeneration) [64,65].

Two recent reports have described children with homozygous truncating mutations leading to total loss of SOD1 enzyme function. Andersen et al. describe a 2-year-old girl, born to consanguineous parents, who developed an upper motor neuron-predominant neurodegenerative phenotype at 6 months of age [26]. Whole exome sequencing revealed homozygous truncating SOD1 mutations at amino acid 112. SOD1 enzyme activity was found to be absent in patient erythrocytes, and 50% of normal in each of the healthy parents erythrocytes. Cultured fibroblasts taken from the index patient would grow in a hypoxic environment with ascorbate, an alternative superoxide scavenger, present, but not without. Park et al. describe a 6-year-old boy with the same homozygous truncating mutation, developing a spastic tetraparesis phenotype from 9 months of age after initial normal development [66]. Cerebellar atrophy was also present in this case. While it cannot be demonstrated from these two cases alone that absence of enzyme activity caused the phenotype, they provide reason to exercise a high level of caution when developing therapies that completely silence SOD1, and strengthen the case for partial silencing approaches in future such as mutant allele-specific silencing.

2. Genetic therapy approaches in mutant SOD1-associated fALS

The divergent effects of mutant SOD1 across many different vital cellular processes (as outlined above, and in Figure 1) provide a case for silencing the SOD1 gene, rather than attempting to ameliorate any one of these processes individually. Targeting SOD1 at the protein level is challenging, as it is highly abundant in cells and can adopt various conformational states with differing affinity for antibodies and small molecules [67]. The relatively mild, non-neurodegenerative phenotype of SOD1 knockout mice has provided the rationale for the relative safety of a cautious gene knockdown approach.

Three broad categories of genetic therapy designed to target SOD1 directly and have shown promise in preclinical models. Two of these, RNA interference (RNAi) and antisense

oligonucleotides (ASOs, together with RNAi referred to here as 'antisense therapies') target SOD1 mRNA, while the use of CRISPR targets the SOD1 genomic DNA directly (**Table 1**). A fourth gene therapy strategy, over-expression of macrophage migration inhibitory factor (MIF), potentially ameliorating SOD1 misfolding, has also shown promising results in animal studies, and is discussed in section 3 [68].

2.1. Gene silencing: RNA interference (RNAi)

RNAi occurs endogenously in eukaryotic cells and serves to negatively regulate gene translation [69,70]. The discovery of this mechanism has revolutionised molecular biology, and has led to intensive interest in the development of RNAi mediated therapies for genetic diseases, cancer and viral infections. The first licensed RNAi-based therapy became available in 2018 for hereditary transthyretin-mediated amyloidosis [71].

In the endogenous RNAi process: microRNA (miRNA)-mediated RNAi relies on the transcription of non-coding genomic DNA to form primary miRNA (pri-miRNA), which is then modified to form pre-miRNA in the nucleus, and subsequently miRNA after export to the cytoplasm. In the cytoplasm, miRNA associates with specialised proteins known as the RNA-induced silencing complex (RISC). The RISC/miRNA complex then binds and cleaves complementary mRNA, blocking translation (and promoting mRNA degradation by RNase). In this system, base mismatches are tolerated, leading to a broad specificity of gene silencing. Up to 60% of human genes are thought to be regulated by this mechanism [72]. One endogenous microRNA, miR-206, has been shown to downregulate SOD1 in mice and dogs, and has reduced expression levels in muscle of sporadic and familial ALS patients [73,74]. MiR-206 is therefore an attractive target for potential therapies and as a biomarker for ALS, but further study is needed [75].

Short interfering RNA (siRNA) operates by a similar mechanism to miRNA, but the specificity of the siRNA/RISC complex is higher, requiring full base complementarity. SiRNA can be introduced into the cell either directly as mature double-stranded duplexes, or expressed in

vectors, most commonly as short-hairpin RNA (shRNA) which is then processed by endogenous cellular machinery and packaged into the RISC complex [76]. Artificial RNAi approaches which depend on endogenous cellular machinery are at risk of causing cell toxicity through over-saturation of these mechanisms, may stimulate immune responses, and cause liver toxicity [76,77]. Careful design and control of the RNAi dose and sequence can help to mitigate this risk [77].

Commonly, RNAi therapies do not penetrate tissues effectively and an inadequate clinical and/or biological response is seen, particularly in tissues such as the central nervous system (CNS), in which the blood brain-barrier has to be overcome. Use of AAV vectors with selective CNS tropism (such as AAV9 and AAV10rh) and intrathecal dosing can help to overcome this [17]. However, delivery using viral vectors further increases the risk of adverse immune responses, with the added risks of genotoxicity and viral persistence in host tissues (Table 1) [17,78]. Despite these risks, the use of AAVs as vectors to deliver gene therapy has led to licensed medications which are safe, well tolerated and highly efficacious in other diseases such as haemophilia A and spinal muscular atrophy (SMA, a degenerative lower motor neuron disease with some features in common with ALS) [79,80]. The use of AAV serotypes with tissue-specific tropisms has facilitated therapeutic design by helping to maximise on-target and reduce off-target effects. An AAV-mediated delivery of an RNAi therapy has yet to be proven as a viable therapy in human disease, although several efforts are ongoing, including for SOD1-associated ALS (see below). The theoretical benefits of such a therapy over ASOs (Section 2.3 below) are considerable, as long-term therapeutic gene knockdown may be obtained using only a single dose, as observed in rodent models [17].

2.2. RNAi-based therapeutic approaches in SOD1-associated fALS

RNAi-based treatment strategies have shown significant promise in preclinical models of mutant SOD1-associated fALS. An excellent 2017 review by van Zundert and Brown on SOD1 silencing

has summarised a wealth of preclinical data pertaining to this area [17]. Therefore, we will focus only salient preclinical data prior to 2017, with updated findings from the past three years in this section.

The first attempt at using RNAi to silence mutant SOD1 dates back to 2003: Ding et al. used siRNA to specifically target the mutated region in a SOD1 mouse model [81]. This attempt was hampered by limited CNS penetrance, and prompted the need to investigate viral vectors as an alternative means for RNAi delivery in the CNS. The first successful attempts using a lentivirus/shRNA-based strategy in mutant SOD1 transgenic mice were carried out in 2005 [82,83]. Both groups demonstrated that the vector distributed well throughout the CNS, causing widespread knockdown of SOD1 and a reduction in motor neuron death. Ralph et al. also demonstrated a 77% increase in median survival of $SOD1^{G93A}$ mice, proving for the first time the exciting therapeutic potential of SOD1 silencing *in vivo.* The translational potential of this important study was, however, limited by the early, pre-symptomatic administration (age 7d), and the mechanism of delivery: via multiple intramuscular injections, relying on retrograde transport via motor axons to reach the CNS. Due to extensive denervation, and greater distances between muscles and the CNS, this approach would likely be impractical in ALS patients and has not been attempted.

Adeno-associated viruses (AAVs) provides a safer alternative to lentivirus, with lower potential for mutagenesis, and a more selective CNS tropism. In 2013, Foust et al. showed that an AAV9, could be used in place of lentivirus to deliver shRNA targeting SOD1 when delivered systemically. The vector was shown to cross the blood-brain barrier and cause widespread SOD1 knockdown in the CNS of $SOD1^{G37R}$ mice. This also conferred a modest survival benefit of 39% [84]. In 2019, it was discovered that when the same viral vector was administered via a novel spinal sub-pial approach just before predicted disease onset at 120 days, $SOD1^{G37R}$ mice did not develop any clinical or pathological evidence of a neurodegenerative phenotype up to age 470 days [85]. Moreover, when this therapy was administered mid-disease at 380d, clinical and

neuropathological decline were attenuated. There were two limitations of this study: firstly, that all mice were sacrificed at 470 days, so longer term survival was not assessed (although a considerable survival benefit vs untreated mice was observed). The reason for this sacrifice was death by cardiac arrest in four of seventeen treated mice. The investigators subsequently demonstrated autonomic instability in $SOD1^{G37R}$ mice in response to grip strength testing as the cause for the deaths: this effect was not attributed by the authors to the treatment. The second limitation is the sub-pial delivery method, which has not yet been attempted in humans (although this was attempted by the authors in non-human primates, with good distribution of the shRNA AAV9 vector throughout the CNS also noted). The safety profile of such a procedure has not yet been established in humans. Patients undergoing this treatment would need to have an invasive neurosurgical procedure, potentially limiting clinical application in frail patients or in those with respiratory muscle weakness.

Recently, a study by Iannitti *et al* demonstrated good efficacy of a clinical trial-ready AAV9-shRNA construct when delivered intrathecally into $SOD1^{G93A}$ mice, resulting in a 44% improvement in survival without off-target effects from sod1 knock-down. This study also demonstrated the effectiveness of CSF SOD1 as a pharmacodynamic biomarker [86].

A preliminary phase I report has recently been published describing the first two administrations of an RNAi-based therapy for SOD1-associated fALS in humans [6]. In this study, a miRNA targeting human SOD1 was incorporated into an AAV10rh vector and delivered intrathecally as a single dose. Previously, the same AAV-miRNA-SOD1 construct was administered to large non-human primates with good safety and SOD1-silencing profiles [87]. In one male patient with a $SOD1^{A5V}$ mutation (associated with rapidly progressive ALS), a reduction in SOD1 relative to expected was observed in post-mortem spinal cord tissue. However, no significant reduction was observed in the CSF, and no definite attenuation of clinical progression was observed. Furthermore, the patient also developed a painful meningoradiculitis associated with

loss of lower limb sensory function, despite concomitant immunosuppressive treatment with steroids [6]. A second patient underwent a more aggressive immunosuppressive regimen prior to administration of the AAV-miRNA-SOD1 vector: meningoradiculitis did not occur in this patient and his ALS symptoms remained largely stable over the subsequent 60-week period (although this patient was homozygous for D91A, associated with slow progression) [6]. This study highlights the need for careful vector design and dosing, and brings into question the applicability of animal models, even large non-human primates, in trials of safety for these complex genetic therapies.

2.3. Gene silencing: antisense oligonucleotides (ASOs)

Prior to the discovery of the endogenous RNAi pathway, it had been observed that administration of short synthetic DNA or RNA-based oligonucleotides (ASOs) can transiently reduce gene expression by binding to mRNA through canonical Watson-Crick base pairing. ASOs can block translation through a variety of mechanisms, such as binding to the 5' UTR region of mRNA and preventing capping, promoting RNase H-mediated degradation, or interfering with mRNA splicing [76]. Chemical modification of the ASO can improve affinity for mRNA, alter tissue penetration, and mediate endogenous RNase activity: these modifications mainly involve substitution of the phosphate-sugar backbone with synthetic alternatives, such as phosphorotioate (PS) or morpholino backbones. The favourable pharmacokinetic and pharmacodynamic properties of modified oligonucleotides must be balanced with the propensity of these synthetic molecules to stimulate inflammatory responses, which can in some cases be predicted by the CpG content [88,89].

ASOs are large, charged molecules: while they cannot easily cross from the systemic circulation into the brain and spinal cord, they have been shown to distribute effectively throughout the CNS when delivered intrathecally, making them an appealing strategy in neurodegenerative diseases [90]. ASOs have now been licensed for several diseases, including cancer, diabetes, viral infections and SMA [91]. Nusinersen (Spinraza) is an ASO that was first licenced for SMA in

2017: it acts by interfering with mRNA splicing, enhancing expression of the survival of motor neuron (SMN) protein from the SMN2 gene copy. Clinically, this results in significantly improved motor function and survival for patients with both the juvenile, and adult forms of the disease [92,93]. Intrathecal ASOs have since entered human trials for other monogenic neurodegenerative diseases, including: *SOD1*-ALS (section 2.4); *C9orf72*-ALS (NCT03626012); Huntington's Disease (NCT02519036, NCT03225846, and NCT03225833), with the common aim of reducing mutant gene expression (additionally ameliorating RNA-induced toxicity in the case of *C9orf72*-ALS) [94]. An early phase trial using an intrathecal ASO to silence the WT *MAPT* gene (encoding the tau protein) in mild Alzheimer's disease is also underway (NCT03186989).

2.4 ASO-based therapeutic approaches in SOD1-associated fALS

The first preclinical trial of an ASO targeting SOD1, ISIS333611, was carried out by Miller et al. in 2006 [95]. This demonstrated effective delivery throughout the CNS in a $SOD1^{G93A}$ rats with a dose-dependent reduction in SOD1 protein and mRNA in neuronal and non-neuronal cells. A modest survival benefit was also noted. The same molecule was later used to establish CSF SOD1 levels as a useful disease biomarker: dose-dependent CSF SOD1 reduction was observed at similar levels to those seen in brain tissue, up to 40% of untreated levels [96]. Miller et al. went on to test ISIS333611 in a phase I study in 2010 [97]. 21 patients participated in this randomised, placebo-controlled single-ascending dose study. Doses up to 3 mg were tested via a single intrathecal infusion over 11.5 hours. While an excellent safety and tolerability profile was established, the treatment failed to significantly reduce CSF SOD1 levels. This was likely to be caused by the small doses tested due to the conservative approach of this first-in-man study.

Clinical trials testing a second ASO, Tofersen (previously BIIB067) commenced in 2015. The initial phase I/II study tested single- and multiple- ascending doses of the drug delivered intrathecally via lumbar puncture. Doses of 20-100 mg were tested, and were safe and well

tolerated over the 12-week dosing period [5]. In the highest multiple ascending dose cohort, 100 mg Tofersen was administered every 28 days. In this group (10 patients vs 12 controls), a statistically significant 36% mean reduction in CSF SOD1 was observed, despite the small size of the trial and the limited power to detect efficacy [5]. Clinical exploratory endpoints including ALS functional rating score, slow vital capacity and muscle power evaluated by hand held dynamometry showed a trend towards slower decline in the treatment group. Slowing of decline was most apparent in patients with rapidly progressive disease. Other exploratory endpoints, blood and CSF levels of neurofilament light and phosphorylated neurofilament heavy, were also found to be reduced from baseline in the treatment group, consistent with a neuroprotective effect. Adverse events included an elevation in CSF white cell count and protein of unclear aetiology in some patients, and lumbar puncture (LP) procedure-associated symptoms. In unpublished reports, myelitis has been observed with Tofersen administration in two patients resulting in sensory and motor deficits. The trial is now in phase III (NCT02623699) and a long-term open-label extension phase (NCT03070119), with completion expected in 2021.

A downside to intrathecal ASO therapy for CNS disease is the need for repeated LPs at frequent intervals for dosing, which is a fairly unique and understudied clinical scenario. In idiopathic intracranial hypertension, patients may require repeated LPs to relieve CSF pressure and tend to feel negatively about this: they demonstrate a high level of anxiety regarding future LPs and frequently report LP-related side effects [98]. While LP-related side effects were observed in most patients enrolled in the phase I/II Tofersen study, these patients anecdotally tolerate LPs significantly better than the general population and experience only mild symptoms of headache and back pain following each procedure. This may possibly be because the intrathecal administration of the study drug in artificial CSF replenishes the CSF removed for analysis, thereby mitigating excessive changes in CSF pressure. Alternatively, the motivation engendered by the poor prognosis of the untreated SOD1-related ALS may promote better pain tolerance. Intrathecal

pumps and reservoirs are technologically advanced and used widely in other conditions [99,100]. These technologies may provide a better-tolerated long-term alternative to repeated lumbar punctures for repeated ASO administration in CNS disease. Furthermore, in leptomeningeal carcinomatosis, where maintaining high CNS concentrations of chemotherapy is crucial, intrathecal delivery of chemotherapy via a surgical reservoir was found to be superior to serial lumbar puncture [99]. However, the downsides of potentially risky surgery may outweigh the benefit of improved tolerability, convenience and potentially superior pharmacokinetic properties in ALS patients and will require careful evaluation.

2.3.Gene editing: CRISPR/Cas9

First described in 2013, the CRISPR/Cas9 gene editing system is derived from bacterial cell defence mechanisms [101,102]. Briefly, it comprises two components: a guide RNA (gRNA), complementary to the region of genomic DNA to be targeted, and a Cas9 nuclease which is directed by the gRNA to form double strand breaks at a precise location. The regions which can be targeted with this system are dictated by the presence of specific nucleotide sequences called protospacer adjacent motifs (PAMs), which occur frequently throughout the genome. After a double strand break, endogenous cell machinery may repair the break using either non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ frequently leads to indel mutations, causing a frameshift and resultant gene knockout [103]. The homology-directed repair mechanism may also be exploited to correct a specific mutation when a template DNA strand is introduced to the cell at the same time. CRISPR/Cas9 has been heralded as a huge breakthrough for future gene therapy: it is hoped that monogenic disease may in future be cured or prevented through correction of mutations in somatic or germline cells respectively.

2.4. CRISPR-based therapeutic approaches in SOD1-associated fALS

A 2017 study by Gaj *et al.* described an AAV9-delivered, CRISPR/Cas9 system targeting the mutant SOD1 gene in $SOD1^{G93A}$ mice [104]. Here, neonatal mice were administered systemically with the AAV9-CRISPR/Cas9 as a one-off dose. Despite widespread delivery throughout the CNS, this resulted in very modest *in vivo* genome editing (0.2 - 0.4%) as measured by indel efficiency using deep sequencing. However, a striking 2.5-fold decrease in mutant SOD1 protein was seen in brain and spinal cord tissue. A 37% delay in disease onset, and a 25% increase in survival time were also observed in treated animals. The discrepancy between indel efficiency and clinical effect remains unaccounted for, but is a common occurrence in CRISPR-mediated genome editing, particularly *in vivo*. The authors postulate that CRISPR interference, a phenomenon whereby the Cas9 nuclease interrupts transcription but does not cleave, may play a role in explaining the difference [105]. They also noted that other *in vivo* knockdown studies using CRISPR/Cas9 have demonstrated a clinical effect that is disproportionate to the measured editing efficiency [104].

A second CRISPR-based technique has recently been deployed in mouse studies to target the SOD1 gene. CRISPR base editors employ CRISPR RNA-guided targeting, but use a mutant Cas enzyme which does not cut DNA, but rather induces single base changes at specific sites without double strand breaks. This can be used to induce premature stop codons at desired sites throughout the genome with fewer off-target effects [106]. CRISPR base editors are too large to package into AAV vectors: Lim et al. used an innovative approach using dual AAV particles encoding a split intein cytosine base editor intrathecally injected into $SOD1^{G93A}$mice. This resulted in a slower rate of muscle atrophy compared to untreated controls and demonstrated proof-of-concept of the potential application of *in vivo* CRISPR-mediated base editing in neurodegenerative disease [107].

In vivo use of CRISPR editing is still very much in its infancy, and unexpected findings as described above are likely to continue to occur. One major consideration is the off-target genomic cleavage, as the system is able to tolerate mismatches between the gRNA and target DNA fragment. This will be particularly relevant if mutations are to be corrected in germline cells in future, as off-

target gene knockouts could have widespread unpredictable effects. Novel CRISPR-based editing approaches such as base editing as described above, and more recently PRIME editing, improve precision, and avoid the need for double stranded breaks, which may lead to safer clinical applicability in future [108]. PRIME editing also obviates the need for a PAM site in the immediate vicinity of target DNA and theoretically allows correction of 89% of known pathogenic mutations in the human genome, if *in vivo* delivery could be achieved.

3. Other treatment strategies: small molecules, peptides, monoclonal antibodies and vaccination

3.1. Small molecules

While gene therapy remains the focus of preclinical and clinical research in SOD1 targeting, a variety of other techniques have also shown promise in early clinical and preclinical studies. Small molecule strategies have largely focussed on preventing misfolding of, or sequestering mutant SOD1, thereby ameliorating toxic gain-of-function in a similar manner to antisense therapies (**Figure 1**). One of these molecules, Arimoclomol, is currently in phase III development at the time of writing (NCT03491462). Arimoclomol promotes normal SOD1 folding in the ER through the induction of heat-shock protein pathways, thereby reducing monomerization and aggregation [109]. Preclinical studies showed significantly improved survival in $SOD1^{G93A}$ mice when initiated early in the disease course (before 75 days). SOD1 aggregation was reduced and muscle function increased in mice regardless of initiation time. Despite the phase I/II studies being carried out on rapidly-progressive mutant SOD1-associated ALS patients, Arimoclomol is hypothesised to be effective in ALS more broadly by promoting proteostasis; the phase III study eligibility criteria have therefore been relaxed to reflect this and now include sporadic ALS in addition to those with confirmed SOD1 mutations [110]. Pyrimethamine, commonly used to treat toxoplasmosis, was identified in an *in vitro* screen to reduce cellular SOD1 levels and has subsequently been shown to

reduce blood and CSF SOD1 in pilot clinical studies [111,112]. The authors also note a possible slowing of disease progression in patients with known rapidly-progressive mutations, despite the open-label design and limited sample size: a phase III trial is awaited.

Another small molecule with a novel pharmacodynamic action was identified by Tsuburaya et al. through *in vitro* screening of 160,000 candidates using fluorescence resonance energy transfer (FRET) to inhibit mutant SOD1 interaction with ER Derlin-1 (**Figure 1**, 'compound #56'). Administration to $SOD1^{G93A}$ mice resulted in a 15% delay in symptom onset and similarly improved median survival by 14%, although there was significant heterogeneity in effect size between treated mice [113].

While mutant SOD1 is ubiquitously expressed, one hypothesis for CNS-specific toxicity is the relative bioavailability of copper in these tissues, and the propensity for SOD1 to misfold in copper-deficient milieu [114]. The copper-containing PET-imaging agent, Cu-ATSM has been shown in several different transgenic mouse model studies to improve survival by 8-25% [114–117]. Mechanistically, Cu-ATSM enhances metalation of CNS SOD1 through the direct transfer of copper ions, leading to a relative abundance of enzymatically active holo-SOD1 protein and a reduction in Cu-deficient SOD1 aggregates [114,118]. In mice, this process was shown to occur preferentially in CNS tissue and not in liver [114]. Strikingly, $SOD1^{G93A}$ mice co-expressing the human copper chaperone for SOD1 protein (which paradoxically have a reduced life expectancy of a few weeks), continuous treatment with CuATSM extended lifespan by 18 months, but ALS symptoms appeared when the treatment was interrupted. The effectiveness *in vitro* of CuATSM was found to be correlated with mutant SOD1 with similarities to WT SOD1, but not with mutants that disrupt metal binding, possibly limiting the scope of clinical application [119]. At the time of writing, a phase II/III study of CuATSM is currently in the recruitment stage following favourable safety profiles in phase I (NCT04082832).

Several recent studies have used rational *in silico* design with the intention of creating small molecules to directly inhibit toxic mutant SOD1 aggregation or mediate its interaction with other proteins. Tryptophan 32 (W32), a solvent-exposed residue which is found in human and primate SOD1 but is not evolutionarily conserved across other species, has recently been hypothesised to play a key role in SOD1 misfolding. Substitution of this residue for a conserved serine residue has been shown to ameliorate motor neuron toxicity in zebrafish relative to human WT and mutant SOD1 [120]. In the same study, *in silico s*creening of previously approved drugs for interaction with this residue identified Telbivudine, a drug used in the treatment of chronic hepatitis B, which rescued SOD1 toxicity in a dose-dependent manner. A second study using a different *in silico* screening method and crystallographic validation identified a phenanthridinone-based compound as inhibiting oxidation of W32 [121]. In a separate study, five compounds were discovered to inhibit $SOD1^{G85R}$ interaction with tubulin *in vitro* possibly also through action on W32 [122].

3.2.Peptides

Macrophage inhibitory factor (MIF), is an native SOD1 chaperone and has been identified as preventing misfolded SOD1 accumulation *in vitro*. The presence of MIF in abundance in peripheral tissues but at low levels in motor neurons has been postulated as a key mechanism of motor neuron toxicity in SOD1-ALS [123]. In a recent study, AAV2/9-mediated over-expression of MIF in CNS tissue in $SOD1^{G93A}$ mice modestly prolonged survival and reduced SOD1 aggregation [68].

Using a novel combined computational and experimental approach, a specific aggregation inhibitor of mutant, but not wild-type SOD1, HTB1M3, was recently developed using a combination of focused and random mutagenesis of a scaffold protein, HTB1, in yeast [124,125]. HTB1M3 has yet to be proven in *in vivo* studies as a viable therapeutic agent.

3.3.Immunotherapy – monoclonal antibodies and vaccination

Conceptually, the use of antibodies to target mutant SOD1 has limitations in that most misfolded SOD1 is found intracellularly. However, evidence that mutant SOD1 propagates extracellularly through a variety of transport mechanisms prompted interest in monoclonal antibodies specific for misfolded SOD1 as a treatment strategy [126]. Two studies of passive immunisation in transgenic mice using monoclonal antibodies specifically targeting misfolded SOD1 modestly improved survival and reduced aggregate formation in $SOD1^{G93A}$ and $SOD1^{G37R}$ mice [127,128]. Interestingly, a greater relative survival benefit was conferred to the slower-progressing $SOD1^{G37R}$ mouse in the recent study by Maier et al., in contrast to what is observed in antisense studies [128].

Active immunisation has been trialled in transgenic mice using both mutant and WT SOD1 with the aim of reducing extracellular mutant SOD1; a similar improvement in survival to that shown by monoclonal antibodies was demonstrated in both cases [129,130]. As with the monoclonal antibody study by Maier et al., these studies also report a significantly greater improvement in the treatment of slowly progressive $SOD1^{G37R}$ when compared with more rapidly progressive $SOD1^{G93A}$ mice [128,131]. It is not yet clear whether the slowly-progressing nature of disease in the $SOD1^{G37R}$ mouse, or the differences in antigen immunogenicity between the $SOD1^{G93A}$ and $SOD1^{G37R}$ proteins explain these differences: further studies in other slowly progressive mouse models would be helpful.

4. Future directions: targeting wild-type SOD1

The future of SOD1-targeting therapies will largely depend on the successes or failures of the clinical trials described above. Due to the relatively small number of patients living with mutant SOD1-associated fALS at any one time, these trials can be hindered by challenges in recruitment. Clinical effect may only be evident over the period of a trial in patients with rapidly-progressing disease, so assessment and validation of biomarkers such as CSF SOD1 and neurofilament levels

may prove to be crucial. Recruiting these rapidly-progressive patients to trial is difficult due to the limited time course of their disease. If the final results of these SOD1-targeting trials prove to be positive, it may provide massive benefit to the small minority of ALS patients carrying SOD1 mutations. However, some investigators have postulated that targeting WT SOD1 in neurodegenerative disease not associated with SOD1 mutation may also prove to be beneficial [17].

4.1. Targeting wild-type SOD1 in sporadic ALS (sALS) and other genetic causes of ALS

A major area of controversy remains regarding the role of WT SOD1 in sALS. The aetiology of sporadic ALS is unknown: it can generally be differentiated pathologically from SOD1-associated fALS by the presence of TDP-43 aggregates in neurons and glia, but the clinical picture is indistinguishable [132]. As discussed in section 1.1, pathological mutations in SOD1 can occasionally be identified in sALS cases. In these instances, it would seem logical that the disease should be treated in the same way as mutant SOD1-associated fALS as above [133]. The role for the WT SOD1 protein in sALS without a SOD1 mutation is less clear. WT SOD1 has been shown to misfold *in vitro*: (i) under conditions of oxidative stress, (ii) in a de-metallated state (iii) in a monomeric form [134,135]. Under these circumstances it has been postulated to play a role in sALS pathogenesis [21]. Oxidised WT SOD1aggregates have been detected using conformation-specific antibodies in spinal cord motor neurons and CSF, and are indistinguishable from those observed in familial SOD1 patients in some studies [136,137]. Formation of these aggregates may be a direct consequence of ER stress [138].

Overexpression of WT SOD1 in mice co-expressing mutant SOD1 leads to an accelerated ALS phenotype, implying that WT misfolding can possibly be triggered by that of the mutant protein [139]. The WT protein may exert this toxicity in a similar way to the mutant protein: for example WT SOD1 also complexes with the anti-apoptotic protein bcl-2 and may mediate toxicity through enhancing apoptosis [131,140]. Furthermore, non-cell autotoxicity mediated by human oligodendrocytes reprogrammed from fibroblasts has been shown to be alleviated *in vitro* when

SOD1 is knocked down in sALS as well as a mutant SOD1-associated ALS, but not C9orf72-associated ALS [52]. There is, therefore, a theoretical case for targeting SOD1 in sALS, but seemingly less so in C9orf72-associated fALS, which may have distinct pathophysiology not involving SOD1. Before SOD1-silencing studies in sporadic patients are attempted, more robust clinical data are needed from familial patients with pathological mutations, for whom there is clearer rationale for targeting the gene.

4.2. Targeting wild-type SOD1 in other neurodegenerative diseases

The role of SOD1 in other neurodegenerative diseases is also less clear: exonic mutations in the gene have so far been linked only to ALS, and the related SOD1-deficiency phenotypes described in Section 1.3. In Alzheimer's disease, mechanistic insights have been gained from the study of patients with Down syndrome (trisomy 21). These patients are at greatly increased risk of early-onset Alzheimer's disease, as they have three copies of the amyloid precursor protein (APP) gene. As SOD1 lies on chromosome 21, expression is also increased, but interestingly, it seems to protect against beta-amyloid mediated neurotoxicity in these patients [141].

Mutations in SOD1 are not associated with Parkinson's disease (PD) [142]. However, a study by Trist et al in 2017 demonstrated the presence of aggregated WT SOD1 in the midbrain structures of post-mortem PD patients, correlating with areas of neuronal loss [143,144]. These aggregates were distinct from the alpha-synucleinopathy usually observed in PD. The SOD1 protein in these aggregates was found to be in a de-metallated state, similar to those associated in hyaline conglomerate inclusions associated with mutant SOD1 fALS. Interestingly, Cu-ATSM has also shown to improve motor and non-motor function in rodent models of PD [115,145]. The mechanism by which this occurs may involve restoring SOD1 metallation, and could reflect a pathophysiological similarity in the role of SOD1 between the two diseases. Alternatively, Cu-ATSM has also been shown to mediate gene expression and ferroptosis in PD, so the mechanism of

action may be distinct in the two diseases [143,146,147]. Early-phase human trials of Cu-ATSM for PD are currently in progress (NCT03204929).

Recently, however, evidence for divergent roles for SOD1 in PD and SOD1-associated ALS has emerged, weakening the case for targeting SOD1 in PD. While defective mitochondrial quality control and mitophagy are implicated in both diseases, the mitophagy-associated protein Miro1 is degraded by fALS-mutant SOD1, yet has impaired clearance in Parkinson's patients and is being considered as a both a biomarker and drug target [43,148,149]. Degradation of Miro1 by mutant SOD1 is dependent on the ubiquitin ligase Parkin [43]. Loss of loss-of function mutations in Parkin are associated with familial PD, whereas Parkin knockdown is protective in SOD1 transgenic ALS mice [150,151].

If SOD1 misfolding is implicated in PD neurodegeneration, it may more likely to occur as a downstream consequence than an upstream cause. Despite this, it may still prove to be a valuable modifiable drug target for Parkinson's disease in the future.

5. Future directions: ideal timing of SOD1 targeting

A common concern for patients and investigators carrying out SOD1 targeting trials is that of timing of therapy. Logic would suggest targeting the toxic mutant SOD1 build-up as early in the disease course as possible would be the most effective strategy, and this is supported by transgenic rodent studies across most SOD1-targeting treatment modalities [17]. On the other hand, the recent reports of infants with absent SOD1 activity and early-onset neurodegenerative phenotype should spark caution in trying to target SOD1 too early in humans [26,66].

SOD1 targeting in asymptomatic SOD1 mutation carriers will require careful study, but may prove very effective if downstream effects of the mutant protein can be curtailed early in the disease course prior to neuronal loss. In the case of Tofersen, the potential benefits of early disease

modification will have to be weighed against the burden of invasive, expensive and potentially risky monthly intrathecal injections in otherwise healthy individuals and should not be undertaken lightly. Restricting future trials of SOD1-lowering therapies in asymptomatic carriers to those with highly penetrant SOD1 mutations associated with rapidly progressive disease would seem prudent.

6. Expert Opinion

The preliminary success of Tofersen in the clinic represents an exciting milestone in the ALS field, fifteen years after SOD1 gene-silencing was proven to be a successful treatment strategy in rodent ALS models. With safe and effective *in vivo* knockdown of CNS SOD1 in humans now achievable, the future merits of pursuing SOD1-targeting may hinge on the successes or failures of the Tofersen phase III trial and open-label extension study, the results of which are highly anticipated. While many other diverse SOD1-targeting strategies have also shown considerable promise in animals, further clinical development is likely to be limited by recruitment due to the low point prevalence of patients with mutant SOD1-associated ALS, coupled with the ongoing trials for Tofersen, the current front-runner. Furthermore, at the time of writing, the ongoing COVID-19 pandemic will undoubtably place a significant strain on recruitment and participation in clinical trials for ALS and in general for the foreseeable future. The delivery of trials requiring frequent, prolonged contact with participants for dosing, such as for Tofersen, pose additional challenges for participants and study sites. The need to reduce in-person dosing visits may expedite the development of intrathecal delivery systems, such as surgical reservoirs, which do not necessitate a hospital attendance for dosing. In this context, the attractiveness of a single-dose SOD1 knockdown strategy theoretically achievable with viral RNAi approaches is further emphasised.

If Tofersen, or other SOD1-lowering therapies do prove effective in slowing disease progression in mutant SOD1 ALS, there may be a rationale for targeting WT SOD1 in sALS patients without confirmed SOD1 mutation, significantly expanding the opportunities for

recruitment and potential patient benefit. Notably, the relaxation of phase III eligibility criteria for the trial of Arimoclomol to include sALS in addition to SOD1-ALS may too provide an enticing glimpse at the translational potential of a SOD1-targeting therapy in a sALS population. A comparison of the responses to SOD1-lowering therapies in SOD1-ALS and sALS may also provide great insight into the pathophysiology of sALS and the role of WT SOD1 in these patients.

In pre-clinical studies, the most significant disease-modifying effect is seen in SOD1-targeting therapies when it is administered as early in the disease course as possible. In a real-world clinical setting, with the exception of cases picked up with genetic screening, SOD1-ALS is a late-onset disease, with the majority of patients presenting after a significant degree of neuronal loss has already occurred. Development of a therapy that is effective in all stages of symptomatic disease will be crucial and design of future preclinical and clinical studies should take this into account.

The significant advancements in the development of SOD1-targeting therapies for ALS, and the recent successes of gene therapy for SMA, highlight that the development of personalised therapies for monogenic causes of neurodegeneration may be within reach: a glimmer of hope for the families devastated by these diseases.

Figure and Table Legends

Figure 1 Schematic representation of key mechanisms by which mutant SOD1 mediates toxicity in motor neurons. Likely sites of action of existing and experimental drugs indicated in red text.

Table 1 Comparison of therapeutic gene-silencing approaches targeting SOD1.

Article highlights:

- **SOD1 was the first gene to be implicated in ALS: mutations in SOD1 are seen in 15-30% of familial, and 1.2-1.5% of sporadic ALS cases.**
- **Most SOD1-ALS is autosomal dominant and associated with a toxic gain-of-function of the SOD1 protein through misfolding, aggregation and disruption of many vital cellular processes: mice lacking SOD1 do not develop a neurodegenerative phenotype.**
- **SOD1 can be effectively knocked down at the mRNA level in humans and animal models using antisense oligonucleotides and RNA interference.**
- **Tofersen, now in phase III development, is an intrathecal antisense oligonucleotide: preliminary evidence suggest it is safe, well-tolerated and may have disease-modifying potential.**
- **Various non-antisense SOD1-targeting strategies have been trialled in humans and animals including small molecules, immunotherapy, and novel CRISPR-based approaches.**
- **Development of SOD1 targeting therapies is likely to be hampered by recruitment due to low point provenance of SOD1-ALS: there is some theoretical basis that targeting SOD1 may be beneficial in ALS without SOD1 mutation and Parkinson's disease**

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