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Therapeutic strategies for *C9orf72* amyotrophic lateral sclerosis and frontotemporal dementia

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Purpose of review

An intronic G₄C₂ expansion mutation in *C9orf72* is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9-ALS/FTD). Although there are currently no treatments for this insidious, fatal disease, intense research has led to promising therapeutic strategies, which will be discussed here.

Recent findings

Therapeutic strategies for C9-ALS/FTD have primarily focused on reducing the toxic effects of mutant expansion RNAs or the dipeptide repeat proteins (DPRs). The pathogenic effects of G₄C₂ expansion transcripts have been targeted using approaches aimed at promoting their degradation, inhibiting nuclear export or silencing transcription. Other promising strategies include immunotherapy to reduce the DPRs themselves, reducing RAN translation, removing the repeats using DNA or RNA editing and manipulation of downstream disease-altered stress granule pathways. Finally, understanding the molecular triggers that lead to pheno-conversion may lead to opportunities that can delay symptomatic disease onset.

Summary

A large body of evidence implicates RAN-translated DPRs as a main driver of C9-ALS/FTD. Promising therapeutic strategies for these devastating diseases are being rapidly developed with several approaches already in or approaching clinical trials.

Keywords

C9ORF72-ALS/FTD, gene therapy, small molecule inhibitors, symptomatic management, therapeutic strategies

INTRODUCTION

A GGGGCC hexanucleotide repeat expansion in the first intron of *C9ORF72* causes the most common forms of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [1,2], genetically linking these two clinically distinct adult-onset neurodegenerative disorders. *C9ORF72* ALS, FTD or both ALS and FTD can occur in individual patients and within families [3]. C9-ALS/FTD patients typically have hundreds to thousands of G₄C₂•G₂C₄ repeats, while shorter tracts of 2–24 repeats are present in unaffected people [1,2]. ALS is characterized by motor neuron loss, muscle atrophy, progressive paralysis and usually death within 2–5 years of onset [4]. In FTD, degeneration of neurons in the frontal and anterior temporal lobes can result in personality changes such as apathy, loss of empathy, disinhibition and executive function deficits [4]. Therapeutic options are limited and there are no current treatment options that substantially change the course of *C9ORF72* ALS or FTD. The standard of

care includes the antiglutamatergic drug riluzole for ALS [5] and the antidepressant fluoxetine or a related compound for FTD [6]. In 2017, the free-radical scavenger drug edaravone was approved for use in ALS patients [7]. Unfortunately, none of these

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KEY POINTS

- Hexanucleotide repeat expansions in the *C9ORF72* gene are the most common known genetic cause of ALS and FTD, a spectrum of debilitating and incurable neurodegenerative diseases.
- Repeat associated non-AUG (RAN) translation, which leads to the production of dipeptide repeat proteins, is a major driver of neuronal injury and disease.
- A number of therapeutic strategies aimed at reducing the impact of expansion transcripts, RAN proteins, nuclear transport deficits or stress granule biology have shown efficacy in preclinical models of disease.
- Approaches aimed at modifying environmental factors and lifestyle are promising complementary avenues for improving the primary care of patients.
- Intense research efforts have resulted in several clinical trials and others are expected to start soon.

treatments improve motor or cognitive deficits; however, riluzole and edaravone have been shown to modestly slow disease progression in some ALS patients.

Similar to other microsatellite expansion disorders [8,9], the *C9ORF72* G₄C₂•G₂C₄ mutation is bi-directionally transcribed and sense G₄C₂ and antisense G₂C₄ expansion transcripts form RNA foci and produce repeat associated non-AUG (RAN) proteins [10–15]. Proposed disease mechanisms include toxic effects of the expansion RNAs [1,16]; toxic effects of sense (poly-GA, GR, GP) and antisense (poly-PR, PA, GP) dipeptide RAN proteins [10,12–14] and haploinsufficiency of the *C9ORF72* protein [17,18]. Overall, RAN proteins, particularly GA, GR and PR, have been shown to be toxic in a number of cell culture and animal models (for reviews [19,20]).

The discovery of the *C9ORF72* expansion as the most common genetic cause of ALS and FTD [1,2] has fuelled an interdisciplinary worldwide research effort to understand the mechanisms and develop therapies for this disorder. *C9ORF72* expansions cause nearly 40% of familial and 6–8% of sporadic ALS cases and nearly 18% of familial and 6% of sporadic FTD cases [21,22] in European populations but are relatively rare in Asia [23]. The relatively large numbers of C9-ALS/FTD patients worldwide, combined with multiple emerging therapeutic strategies have positioned C9-ALS/FTD well for breakthrough therapy development. Emerging therapeutic strategies include targeting and removing the expanded repeats; degrading, or preventing expression of expansion transcripts; reducing toxic RAN proteins; and modulating downstream affected pathways

including nucleocytoplasmic transport and stress granules (Fig. 1). These strategies and additional efforts to understand key molecular and physiological changes that trigger disease may provide insights that will lead to better disease management and help stratify the inclusion of the most informative patients for clinical trials.

TARGETING EXPANSION TRANSCRIPTS FOR DEGRADATION

Sense *C9ORF72*-repeat transcripts

Almost immediately following the discovery of the *C9ORF72* expansion mutation, efforts to develop antisense oligonucleotide (ASO) drugs to knock-down the repeat expansion RNAs began. These nucleic acid based drugs are chemically modified, and in some applications take advantage of the nuclear RNase H1 pathway to degrade double-stranded sequences that form when ASOs bind to targeted gene transcripts [24]. For *C9ORF72*, specific ASOs were shown to selectively reduce sense G₄C₂ RNA foci in patient cells without reducing the levels of *C9ORF72* mRNA [11,16]. BAC transgenic mice treated with single-dose ASOs that selectively target sense expansion RNAs but not mRNAs encoding *C9ORF72* protein, decreased sense RNA foci and sense DPRs and improved behavioural abnormalities [25]. Together, these results paved the way for a phase I clinical trial to test the safety, tolerability and pharmacokinetics of the Ionis/Biogen BIIB078 ASO in adults with *C9ORF72* ALS (NCT03626012).

More recently, stereopure ASOs were shown to increase RNase H activity *in vitro* and *in vivo* compared with stereorandom ASOs [26]. A lead candidate stereopure ASO showed selective degradation of sense G₄C₂ expansion containing transcripts without reducing the variant 2 isoform which lacks the repeat. Treatment of BAC transgenic mice with these ASOs reduced RNA foci and RAN GP proteins but not *C9ORF72* protein levels. In addition, these oligonucleotides selectively protected iPSC-derived motor neurons harbouring *C9ORF72*-expansion mutations from glutamate-induced toxicity [27]. In an alternative approach, miRNAs targeting sense *C9ORF72*-repeat transcripts using adeno-associated virus serotype 5 (AAV5) delivery, reduced levels of sense expansion transcripts and RNA foci in FTD iPSC-derived frontal brain-like neurons and BAC transgenic mice [28]. Interestingly, AAV-mediated miRNA-depletion of SOD1 was recently reported in two patients with SOD1-ALS [29], providing proof-of-concept data that intrathecally delivered microRNAs can be used as a potential treatment strategy for ALS.

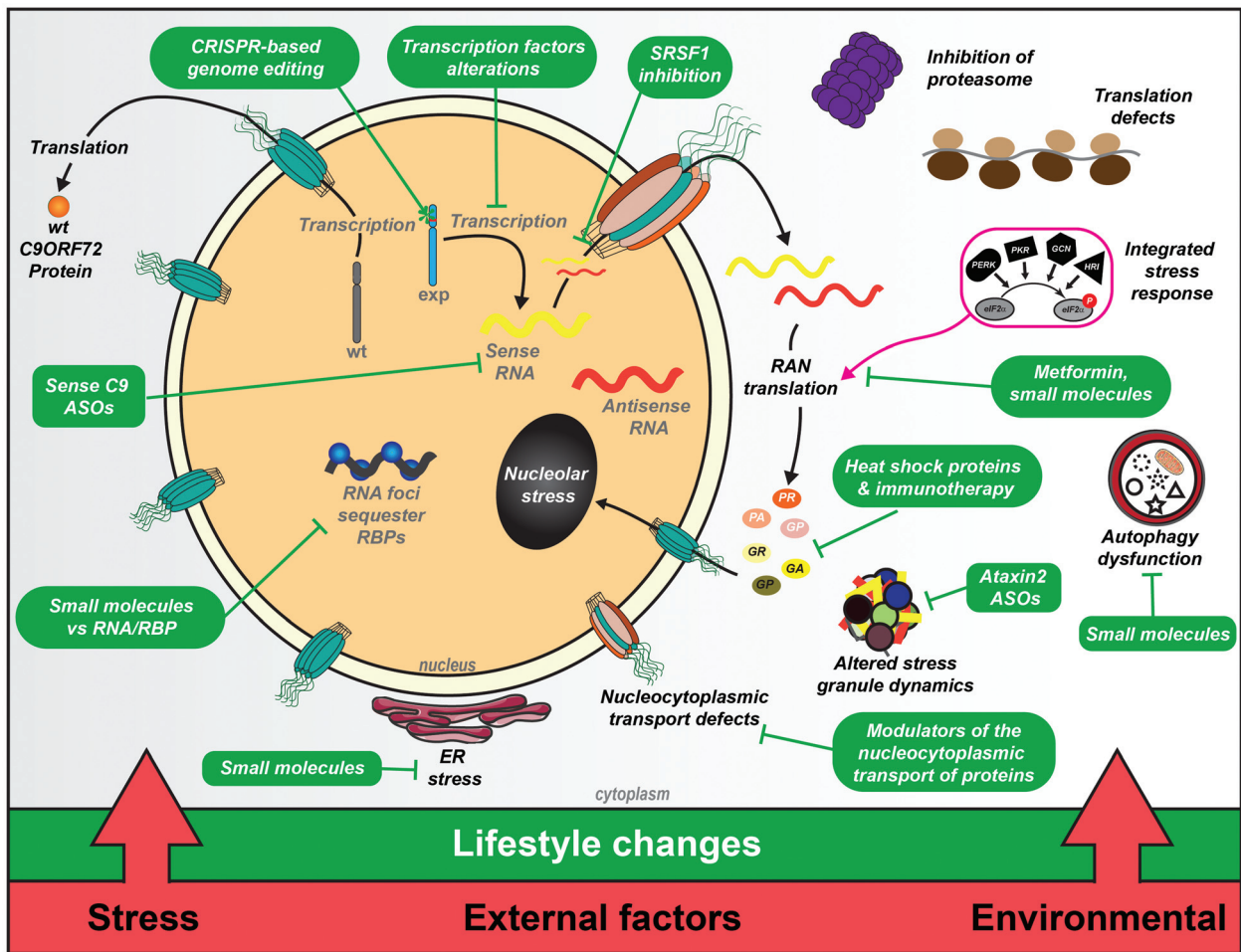


FIGURE 1. Cellular consequences and therapeutic approaches for C9ORF72-ALS/FTD. C9-ALS/FTD affects a wide variety of downstream cellular pathways with different therapeutic approaches (green boxes) targeting pathogenic C9 RNAs and RAN DPRs as well as downstream pathways. External factors (bottom red bar) can also influence cellular events and combined with lifestyle changes (bottom green bar) may affect disease onset and progression. Some content modified from Servier Medical Art (smart.servier.com) under creative commons license.

Inhibiting transcription of C9-expansion transcripts

Several different strategies are being actively pursued to decrease the transcription of C9-repeat expansion transcripts. For example, SUPT4H, SUPT5H and RNA polymerase II associated factor 1 complex (PAF1C) are transcription factors that play important roles in the elongation of RNAs containing expanded repeats [30,31]. Decreased expression of SPT4 encoded by *SUPT4H* was shown to decrease the levels of sense and antisense C9ORF72 expansion transcripts and GP-RAN proteins in *Caenorhabditis elegans*, *Drosophila* and iPSC-derived models of C9ORF72-ALS/FTD and to ameliorate neurodegenerative phenotypes in *Drosophila* [32]. Similarly, depletion of PAF1C reduced expression of G₄C₂ expansion RNAs and poly (GR) in a *Drosophila* model [31]. Although interesting, the

therapeutic potential of these strategies may be limited by off-target effects [33*].

In other approaches, CRISPR-Cas9 deletion of the promoter region driving expression of the C9ORF72 repeat-containing transcript isoforms 1 and 3 led to the efficient reduction in expression levels of all three sense DPRs in C9-ALS patient-derived motor neurons [34*]. Pinto *et al.* [35] showed targeting C9ORF72 expansions at the DNA level using deactivated Cas9 (dCas9) efficiently inhibits transcription and reduced the levels of GP RAN protein in reporter cells. Similarly, use of RNA-targeting deactivated-Cas9 (RCas9) allows degradation of C9ORF72 expansion transcripts in cell models [36], although it is possible that this strategy also blocks transcription by binding of the dCas9-PIN-gRNA to the DNA repeats as in dCas9 strategy.

Although there is great hope that decreasing the levels of G₄C₂ expansion transcripts will be sufficient to improve disease, several studies suggest that it will be important to increase efforts to also target antisense transcripts. For example, antisense RNA foci preferentially accumulate in regions of disease in a *C9ORF72* BAC transgenic mouse model suggesting antisense expansion RNAs or RAN proteins may be more toxic than their corresponding sense products [37]. Additionally, ASOs that target sense *C9ORF72* expansion transcripts did not correct widespread transcriptomic defects found in patient-derived cells [11], suggesting strategies targeting both transcripts may offer the best outcomes.

IMMUNOTHERAPY STRATEGIES TARGETING RAN PROTEINS

There is strong evidence that RAN DPRs, particularly GA, GR and PR, are one of the main drivers of disease (for reviews [19,20]) and hence decreasing their levels is an attractive therapeutic approach. Several immunotherapy approaches have focused on the GA RAN proteins. In patient fibroblasts and primary neurons, α -GA antibodies reduced GA aggregate formation and blocked aggregate seeding activity of cerebellar extracts from *C9ORF72* autopsy tissue [38]. Vaccination of GA-overexpression mice with ovalbumin-(GA)₁₀ peptides elicited the production of α -GA antibodies, lowered GA protein levels and prevented microglial activation and motor deficits [39[■]]. Using BAC transgenic mice that express sense and antisense transcripts and multiple types of RAN proteins, Nguyen *et al.* [40[■]] showed passive immunotherapy with α -GA antibodies improved behavioural deficits, increased survival and decreased neuroinflammation and motor neuron loss. These peripherally injected antibodies crossed the blood brain barrier and co-localized with GA protein aggregates. Glycosylation of the Fc antibody region was important for cell entry and GA proteins were reduced in a TRIM-21-, proteasome-, and autophagy-dependent manner [40[■]]. In addition to reducing GA, the α -GA1 treatment surprisingly also reduced GP and GR proteins, likely through increased proteasome function. No changes in sense or antisense RNA levels or foci were observed in α -GA1 treated mice providing strong support that RAN proteins and not RNA gain of function effects drive C9-ALS/FTD [40[■]].

DECREASING RAN PROTEIN LEVELS

Several groups have shown that activation of the integrated stress response (ISR) and increased p-eIF2 α levels increase RAN translation [41–44,45[■]].

Zu *et al.* [45[■]] showed G₄C₂ and other repeat expansion RNAs activate ISR protein kinase R (PKR) and that PKR inhibition dramatically reduces RAN protein levels. Zu *et al.* [45[■]] went on to show inhibition of PKR using AAV-delivered dominant negative PKR-K296R or the FDA-approved drug metformin, decreased p-PKR and RAN protein levels and improved behaviour and neuropathology in C9 BAC transgenic mice without changing sense or antisense transcript levels. There is an active clinical trial to test the safety of metformin in *C9ORF72* ALS patients and its effects on RAN protein levels (NCT04220021). Inhibition of the SRSF1-dependent nuclear export of both sense and antisense *C9ORF72*-repeat transcripts and subsequent RAN translation was also reported as a promising gene therapy approach in preclinical models including patient-derived motor neurons and *Drosophila* models of disease [46].

Other strategies to reduce RAN protein levels include stimulating their clearance. For example, heat shock protein family B member 8 (HSPB8) has been shown to promote autophagy-mediated removal of several misfolded C9 RAN proteins from motor neurons [47]. Although the therapeutic potential of this approach is uncertain, clearance of protein aggregates could have applications for a wide variety of neurodegenerative diseases. Taken together, these data support the therapeutic potential of targeting RAN translation and RAN proteins for C9-ALS/FTD as well as other RAN protein associated disorders.

TARGETING THE GENOMIC *C9ORF72* HEXANUCLEOTIDE-REPEATS

Correcting the GGGGCC•GGCCCC repeat expansion mutation should theoretically address all deleterious mutation effects, including effects from sense and antisense RNAs and DPRs. Current gene editing techniques have focused primarily on clustered regular interspaced short palindromic repeats (CRISPR)-associated (Cas) systems, although application to C9-ALS/FTD has so far been limited [48,49[■]]. In iPSC-cells, CRISPR/Cas9 editing and homology-directed repair (HDR) replacement of the expansion with a wildtype repeat resulted in restoration of *C9ORF72* gene expression and methylation and reduced intron retention and downstream pathogenic phenotypes [49[■]]. In iPSC-derived motor neurons, CRISPR/Cas9 correction abolished GluA1 AMPA receptor (AMPA) mediated excitotoxicity [48]. Targeting regions outside the repeat or the entire *C9ORF72* gene have also been tested with varying degrees of success. For example, deletion of a portion of the upstream *C9ORF72*

promoter prevents the production of exon 1a expansion containing transcripts and the activation of neurodegenerative pathways [34[■]]. Unfortunately, this approach does not prevent expression of antisense RNA and associated antisense DPRs, which likely contribute to disease. Although correcting the expansion mutation seems to be a straight-forward idea, adequate delivery to affected tissues/cell types and the accuracy of emerging CRISPR based approaches will be critical for effective therapy development.

STRESS GRANULES AND NUCLEOCYTOPLASMIC TRANSPORT

TDP43 plays important roles in transcriptional regulation, alternative splicing of pre-mRNAs, axonal transport of mRNAs, translational regulation and miRNA processing. TDP-43 also associates with stress granules [50], which constitute dynamic membrane-less organelles that promote cell survival by halting translation of nonessential mRNAs in response to cellular stress [51]. Stress granules are composed of RNA and RNA-binding proteins with low complexity domains (LCDs) that mediate liquid-liquid phase separation (LLPS). Mutations in the LCDs domains of TDP43, ataxin-2 and other RNA-binding proteins involved in ALS/FTD stimulate their self-assemblies leading to the formation of persistent cytoplasmic stress granules that leave aggregated proteins that may contribute to disease [52]. Interestingly, arginine-rich C9ORF72 DPRs impair stress granule assembly dynamics by undergoing LLPS, further inducing the phase separation of stress granule proteins [53]. Overexpression of GFP-polyGR proteins impairs stress granules and protein translation in mice deficits [54]. TDP-43 proteinopathy, aggregation of stress granule proteins (G3BP1, ataxin-2), nucleocytoplasmic defects, neuronal loss and motor/cognitive deficits were observed in an AAV-driven overexpression mouse model of expanded G₄C₂ repeats [55]. DPRs also promote nucleocytoplasmic transport disruption by stimulating the recruitment of nucleocytoplasmic transport proteins to stress granules [56]. In fact, many nucleocytoplasmic transport factors are localized to stress granules when exposed to stressors or mutant proteins implicated in ALS pathogenesis, leading to impaired nucleocytoplasmic transport [56].

TARGETING NUCLEOCYTOPLASMIC TRANSPORT DEFICITS

In 2015, Zhang *et al.* [57] demonstrated increased nuclear export in C9ORF72 ALS iPSC models showed abnormal cytoplasmic RanGTPase accumulation.

RanGTPase is important in nucleocytoplasmic protein transport (reviewed in [58]). Abnormal expression and localization of nuclear pore proteins are found in C9ORF72 autopsy tissue and patient-derived iPSCs [57,59]. Modulating the expression of nuclear pore proteins or transport-associated proteins affects G₄C₂ expansion transcripts and arginine containing RAN protein toxicity [57,60,61]. Overexpression of importin or inhibition of nuclear export with RNA inhibition of (Exportin 1) XPO1 or pharmacologically ablating XPO1 function using KPT-276 rescued C9ORF72 toxicity in the C9ORF72 fly model [57]. Another XPO1 inhibitor, KPT-350, designed by Karyopharm Therapeutics and acquired by Biogen (BIIB100), has been used in preclinical studies of many neurological diseases and demonstrated neuroprotective and anti-inflammatory roles [62,63,64[■],65].

TROPHIC SUPPORT SUPPLEMENTATION

Neurotrophic factors (NTFs), a family of biomolecules that support neuronal growth, survival and differentiation, have been explored for decades as therapeutic strategies for neurodegenerative diseases [66[■]], including ALS. Various NTFs have been tested in preclinical rodent models of SOD1-ALS, including brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF). Small molecule agonist of the BDNF receptor [67], VEGF injections [68,69], lentiviral and AAV-mediated delivery of NTFs [70–73] and stem cell therapy of NTF secreting cells [74–82] have shown promise in SOD ALS models. Despite these promising results, including several ongoing clinical trials [66[■]], there have been no direct studies looking at NTF therapeutics in C9-ALS/FTD. Although some NTF clinical trials in ALS likely include C9-ALS/FTD participants, it will be important, given the unique disease mechanisms of C9-ALS/FTD, to directly examine NTF specifically in the C9 context.

PREVENTION AND FUNCTIONAL MANAGEMENT

For C9-ALS/FTD, there is a relatively long period of apparent good health prior to disease onset, which most often occur in the fourth or fifth decade of life, and extending this period of good health has been gaining considerable attention. Both preclinical animal studies and human studies demonstrate that moderate exercise regimens improve functionality and ameliorate disease symptoms for ALS in general (reviewed in [83]). However, the role of exercise is complex. In a retrospective study, patient-reported

exercise history was inversely correlated with age-of-onset in *C9ORF72* but not other forms of ALS [84], although additional studies that examine the impact of specific types of exercise will be important. Targeted training may be key. In a randomized, sham-controlled clinical trial, Plowman *et al.* [85,86[■]] showed that expiratory muscle-strength training is well tolerated in ALS patients and improved bulbar function in a longitudinal *C9ORF72* case-study and also in larger cohorts of genetically undefined ALS patients. Longitudinal studies suggest that lifestyle modifications (e.g. smoking cessation, maintaining a healthy BMI) at a younger age may lower the risk of developing ALS [87[■]]. Recent studies have identified *C9ORF72* expansions in 1.6% ($n=8/487$) of cases with possible idiopathic normal pressure hydrocephalus (iNPH) but not in control cases ($n=0/432$) aged more than 65 years. Clinically significant shunt response was detected in six out of seven shunted *C9ORF72* expansion carriers. Additional studies are needed to understand the frequency of NPH in *C9ORF72* expansion carriers and the potential utility of shunts to drain excess cerebrospinal fluid in these patients. Patient lifestyle, co-morbid diseases and environmental factors (Fig. 1) can influence cellular events, making it important to study and understand external factors that may influence disease. Dietary studies specific to C9-ALS/FTD are rare, although a larger study focused on ALS in general has demonstrated that increasing fruit and vegetable associated fibre, antioxidants and carotenes was associated with improved function [88]. The results of many of these broader ALS studies are complicated by the complex genetics and phenotypic presentation of ALS, increasing the call for preventive and lifestyle studies that focus on *C9ORF72* or other single ALS mutations.

STRATIFICATION AND EFFICIENCY IN CLINICAL TRIALS

Although there are multiple therapeutic approaches for C9-ALS/FTD in the preclinical pipeline, there are only two C9-ALS/FTD specific clinical trials registered in ClinicalTrials.gov: an ASO-based clinical trial of BIIB078 (Biogen), which targets *C9ORF72* expansion containing transcripts for ASO-induced RNase H-mediated degradation (NCT03626012); and a clinical trial (NCT04220021), to test the safety and tolerability of metformin in *C9ORF72* ALS patients and the drug's ability to decrease RAN protein levels. Several additional trials are open to but not specific to C9-ALS/FTD patients. These include a phase 1 clinical trial of BIIB100 to reduce excessive nuclear export (NCT03945279) in C9 and

other ALS patients, will examine the safety, tolerability, pharmacokinetics (PK) and pharmacodynamics (PD) of the drug. A phase 2 clinical trial examining the safety, tolerability, PK and PD of AL001, a recombinant human antihuman sortilin (SORT1) monoclonal IgG1 antibody in FTD patients with either granulin or *C9ORF72* mutations (NCT03987295). Sortilin is a type I membrane glycoprotein involved in progranulin trafficking that is expressed in the central nervous system [89]. Frontotemporal degeneration can be caused by mutation in the progranulin (*GRN*) gene or the *C9ORF72* hexanucleotide expansion repeat and there are rare patients with mutations in both [90]. More recently, Wave Life Sciences has been reported to seek regulatory approval for WVE-004, an investigational stereopure ASO targeting the expansion transcript of C9-ALS/FTD [27[■]]. It is interesting to note the current batch of clinical trials, which focus on different pathogenic pathways, could potentially be used together.

CONCLUSION

Despite the mechanistic and clinical complexity of *C9ORF72* ALS/FTD, intense research efforts over the 10 years since the expansion mutation was identified have led to a remarkable number of novel therapeutic approaches in preclinical and clinical trial stages. The breadth and diversity of these approaches provide hope for C9-ALS/FTD patients, who currently have limited therapeutic options focused on supportive care. The pace of research focused on the root causes of this disease has been remarkable and is likely to accelerate and uncover additional new therapeutic targets and treatment strategies that will significantly impact C9-ALS/FTD and the larger family of repeat expansion disorders.

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Conflicts of interest

G.M.H. is an inventor on patents granted in the USA (US10801027B2) and Europe (EP3430143B1) for the use of inhibitors of SRSF1 to treat neurodegenerative disorders (WO2017207979A1). L.P.W.R. is an inventor on patents and pending patents related to RAN translation and the use of metformin and PKR inhibition to treat RAN protein disorders. The authors declare no other relationships, conditions or circumstances that present a potential conflict of interest.

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