Therapeutic strategies for \textit{C9orf72} amyotrophic lateral sclerosis and frontotemporal dementia

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\textbf{Purpose of review}
An intronic G\textsubscript{4}C\textsubscript{2} expansion mutation in \textit{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.

\textbf{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 G\textsubscript{4}C\textsubscript{2} 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.

\textbf{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.

\textbf{Keywords}
\textit{C9ORF72}-ALS/FTD, gene therapy, small molecule inhibitors, symptomatic management, therapeutic strategies

\section*{INTRODUCTION}
A GGGGCC hexanucleotide repeat expansion in the first intron of \textit{C9orf72} causes the most common forms of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) \cite{1,2}, genetically linking these two clinically distinct adult-onset neurodegenerative disorders. \textit{C9orf72} ALS, FTD or both ALS and FTD can occur in individual patients and within families \cite{3}. C9-ALS/FTD patients typically have hundreds to thousands of G\textsubscript{4}C\textsubscript{2}\textsubscript{\cdot}G\textsubscript{3}C\textsubscript{4} repeats, while shorter tracts of 2–24 repeats are present in unaffected people \cite{1,2}. ALS is characterized by motor neuron loss, muscle atrophy, progressive paralysis and usually death within 2–5 years of onset \cite{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 \cite{4}. Therapeutic options are limited and there are no current treatment options that substantially change the course of \textit{C9orf72} ALS or FTD. The standard of care includes the antiglutamatergic drug riluzole for ALS \cite{5} and the antidepressant fluoxetine or a related compound for FTD \cite{6}. In 2017, the free-radical scavenger drug edaravone was approved for use in ALS patients \cite{7}. Unfortunately, none of these

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\textit{Curr Opin Neurol} 2021, 34:748–755

DOI:10.1097/WCO.0000000000000984

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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 bidirectionally transcribed and sense G4C2 and antisense G2C4 expansion transcripts form RNA foci and produce repeat associated non-AUG (RAN) proteins [10–14] 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.

### 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.

### 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 knockdown 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 1 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 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.
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 G_4C_2 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-, proteosome-, and autophagy-dependent manner [40]. In addition to reducing GA, the α-GA1 treatment surprisingly also reduced GP and GR proteins, likely through increased proteosome function. No changes in sense or antisense RNA levels or foci were observed in α-GA1 treated mice providing strong support that targeting both transcripts may offer the best outcomes.

**DECREASING RAN PROTEIN LEVELS**

Several groups have shown that activation of the integrated stress response (ISR) and increased p-eIF2α levels increase RAN translation [41–44,45]. Zu et al. [45] showed G_{4}C_{2} 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 (AMPAR) 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
impaired nucleocytoplasmic transport [56]. 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 [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 iPSN 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 iPSNs [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 occurs 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-strengthening 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 carotenoids 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 EFFICACY 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.

**Acknowledgements**

L.P.W.R. and G.M.H. express their gratitude to ALS patients and their families for participating in research and for donating biological samples to the Center for NeuroGenetics at the University of Florida or the NIHR Sheffield Biomedical Research Centre: Translational Neuroscience for chronic neurological disorders (IS-BRC-1215-20017).

**Financial support and sponsorship**

L.P.W.R. gratefully acknowledges funding from the National Institutes of Health (ROI NS098819, R37NS040389 and PO1-NS058901), Target ALS, ALS Association, Packard Center, Myotonic Dystrophy Foundation, Department of Defense (W81XWH1910654) and Muscular Dystrophy Association for support. GMH thanks the Medical Research Council (MRC)
Motor neuron disease

New Investigator research grant MR/R024162/1 and the Biotechnology and Biological Sciences Research Council (BBSRC) grant BB/S005277/1.

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 KRISP1 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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