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Amyotrophic lateral sclerosis caused by hexanucleotide repeat expansions in C9orf72: from genetics to therapeutics




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## Genetic Amyotrophic Lateral Sclerosis 3

# Genetic ALS caused by hexanucleotide repeat expansions in *C9orf72*

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**GGGGCC repeat expansions in *C9orf72* are a common genetic cause of amyotrophic lateral sclerosis in people of European ancestry; however, substantial variability in the penetrance of the mutation, age at disease onset, and clinical presentation can complicate diagnosis and prognosis. The repeat expansion is transcribed into repetitive RNAs and translated into dipeptide repeat proteins, and both accumulate in the cortex, cerebellum, and the spinal cord. In addition, neuropathological aggregates of phosphorylated TDP-43 are observed in motor cortex and other cortical regions, and in the spinal cord of patients at autopsy. *C9orf72* repeat expansions can also cause frontotemporal dementia. The GGGGCC repeat induces a complex interplay of loss-of-function and gain-of-function pathological mechanisms. Clinical trials using antisense oligonucleotides to target the GGGGCC repeat RNA have not been successful, potentially because they only target a single gain-of-function mechanism. Novel therapeutic approaches targeting the DNA repeat expansion, multiple repeat-derived RNA species, or downstream targets of TDP-43 dysfunction are, however, on the horizon, together with the development of diagnostic and prognostic biomarkers.**

## Introduction

Amyotrophic lateral sclerosis (ALS) is characterised by the progressive degeneration of upper and lower motor neurons, leading to motor dysfunction and, eventually, to respiratory failure. While most patients with ALS do not have other relatives with the disease (and their disease is then categorised as non-familial), about 10% of patients have familial ALS. In these cases, a genetic mutation has likely been inherited. The most common genetic cause of ALS is a mutation that consists of at least 30 repeats of the hexanucleotide GGGGCC in the *C9orf72* gene. In addition to ALS, *C9orf72* mutations also cause frontotemporal dementia. This mutation is one of the many DNA repeat expansions that have been identified to cause neurological diseases. The discovery of this expanded repeat mutation in 2011[1,2] opened a new field of *C9orf72* research in ALS and frontotemporal dementia focused on unravelling mechanisms and finding biomarkers, and on developing treatments to prevent clinical signs and symptoms in carriers of *C9orf72* mutations.

This third paper in a Series on genetic ALS aims to summarise the remarkable research progress that has taken place in the past 14 years, with the characterisation of the clinical and pathological hallmarks of *C9orf72*-associated ALS (*C9orf72*-ALS), the unique molecular aspects of the repeat expansion, and the complex array of underlying mechanisms. We also address how this wealth of information is now being translated into novel biomarkers to aid in diagnosis and prognosis, and review the treatment approaches that are in development, including the first clinical trials in carriers of *C9orf72* repeat expansions.

## The GGGGCC repeat expansion

The GGGGCC repeat expansion implicated in *C9orf72*-ALS is located on the short arm of chromosome 9 in the *C9orf72* genomic region (GGGGCC at chr9:27573529-27573534, build hg38). Neurologically healthy individuals carry between two and 23 GGGGCC copies in this region; however, patients with *C9orf72*-ALS generally have one chromosome 9 with hundreds to thousands of GGGGCC copies (eg, they are heterozygote carriers of the repeat expansion).[1,2] An arbitrary pathogenic cutoff is set at 30 copies, but intermediate repeats in the range of 24 to 30 copies can still confer some disease risk.[3] Insufficient data are available to establish pathogenicity in individuals with repeat lengths between 30 and around 100 copies. From the locus, multiple *C9orf72* mRNA transcripts are generated of which three variants have been best characterised (V1, V2, and V3). For V2, the repeat is in the promoter region of *C9orf72*, while in V1 and V3 the repeat is in the first intron and can thus be transcribed. Translation of these major transcripts is predicted to result in short and long protein isoforms, yet only the long isoform has been consistently detected in human brain using validated tools (figure 1).[4,5]

Most individuals who carry repeat expansions share a small number of haplotypes,[6,7] which suggests a founder effect. Most expansions occur on the so-called Finnish founder haplotype (characterised by the T-allele of rs3849942), which has been refined to a sub-haplotype containing additional risk alleles (A-allele of rs147211831 and C-allele of rs117204439).[8] While still within the healthy range, these founder haplotypes carry a greater number of repeat units (ie, median of 12 repeat units on the sub-haplotype versus median of two repeat units on the non-founder haplotypes), possibly increasing the

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This is the third paper in a **Series** of papers on Genetic Amyotrophic Lateral Sclerosis

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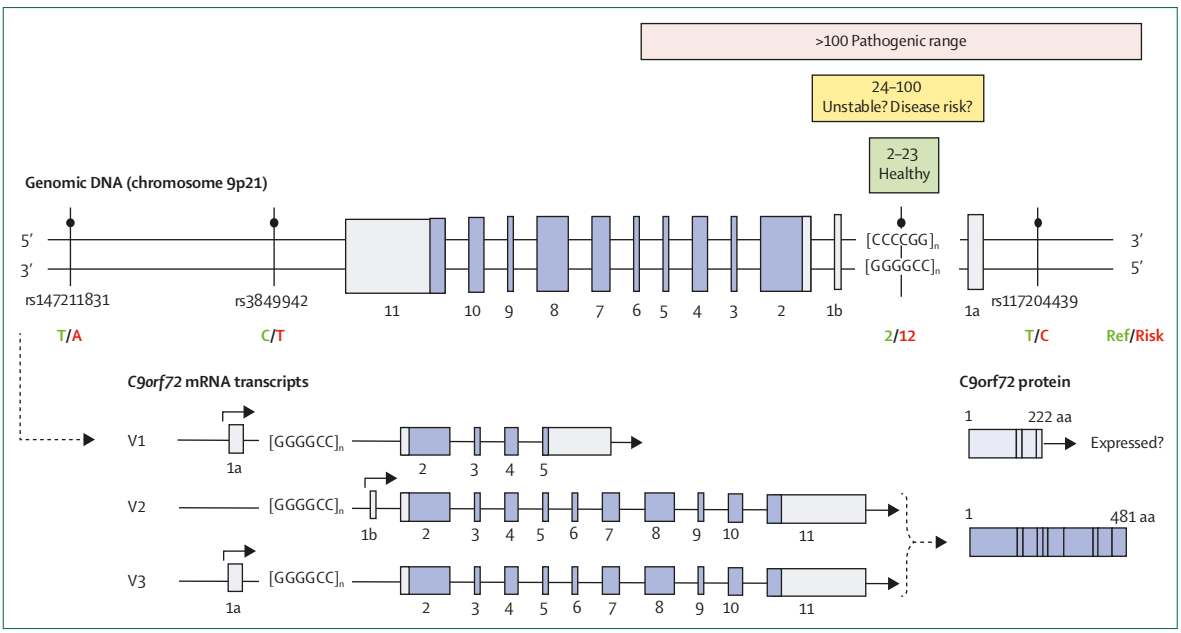


***Figure 1: C9orf72* gene structure**
The chromosome 9p21 genomic locus contains the *C9orf72* gene, which has 11 exons and the $(CCCCGG)_n$ repeat located between exons 1a and 1b. Coding exons are shown in blue and non-coding exons are grey Key genetic variants defining the risk founder haplotype (rs147211831, rs3849942, and rs117204439) are shown relative to the *C9orf72* genomic locus, with their risk alleles shown in capital in red and the reference (non-risk) alleles shown in green. Note that the founder haplotype can have a variable number of repeat units, with a median of 12 repeat units. The number of repeat units determines the pathogenic nature of the repeat. Most studies use an arbitrary cutoff for pathogenicity at 30 repeats; however, most neurologically healthy individuals carry repeats with less than 24 repeats, whereas repeats between 24 and 100 might confer an increased disease risk. Repeats of more than 100 are considered pathogenic. Alternative splicing generates three transcripts (V1–V3). The repeat is located in the first intron of V1 and V3, and in the promoter region of V2. Translation of V2 or V3 generates a C9orf72 protein isoform of 481 amino acids. V1 is predicted to encode a shorter C9orf72 protein isoform of 222 amino acids, but its relevance remains unclear. Ref=reference allele. Risk=risk allele.

likelihood of conversion to a pathological length in future generations. That said, intergenerational de novo expansions from a healthy to a pathogenic size have not been documented and the threshold for instability might lie between more than 30 and around 100 repeats.[9]

## Genetic diagnosis and clinical correlations

Although PCR-based approaches can be used to establish the presence of an expanded *C9orf72* repeat,[10] Southern blots have traditionally been used to estimate its length. These studies revealed that the repeat expansion is somatically unstable, with substantial variability between and within tissues.[11] This variability is exemplified by descriptions of patients with *C9orf72*-ALS or *C9orf72*-associated frontotemporal dementia (*C9orf72*-FTD) who harbour around 100 repeats in blood, but thousands of repeats in the brain.[9,11] Occasionally, individuals who had small expansions in blood but much longer expansions in post-mortem brain tissue, but without clinical symptoms, have been reported, perhaps because of brain mosaicism, as only a fraction of their brain cells carried expanded alleles.[12] These cases underscore the difficulty in defining a pathogenic cutoff size for the repeat, especially based on blood measurements alone. Nevertheless, the length of the repeat expansion has been associated with age at disease onset, age at blood or brain sample collection, and survival time after disease onset.[11,13,14] Several studies also suggest that the expansion can contract in successive generations,[14,13] particularly with paternal transmission.

*C9orf72* repeat expansions can also be detected in short-read sequencing data using specialised tools.[15] However, to obtain highly accurate length and sequence information, and establish whether and to what extend the repeat is methylated (methylation might serve as a disease modifier), long-read sequencing technologies are needed.[16,17] Long-read sequencing (panel 1) in cerebellar tissue from 28 patients with *C9orf72*-ALS or *C9orf72*-FTD showed a unique length and composition of the repeat in nearly every single DNA molecule examined , whereby the expanded allele mainly contained the GGGGCC motif, with occasional interruptions by other motifs.[18] Quantitative studies on the degree of *C9orf72* repeat methylation have not yet been done. Future studies should elucidate whether methylation of the repeat expansion, possibly in addition to length and sequence composition, might contribute to the phenotypic heterogeneity in *C9orf72* expansion carriers.

### Phenotypes and incomplete penetrance

The *C9orf72* repeat expansion is a relatively common mutation in people of European ancestry, accounting for about a third of patients with familial ALS and a quarter

of patients with familial frontotemporal dementia, in addition to 5–6% of patients with apparently non-familial ALS or frontotemporal dementia.[19–21] The *C9orf72* expansion is much less common in other ancestral populations, such as those from Africa, Asia, and Latin America, in line with the near absence of the founder haplotype in these populations.[19] The inheritance pattern of the mutation is autosomal dominant with incomplete penetrance, which is estimated to be as low as 20–50% in some studies,[22] but clearly variable between families.[23] This low penetrance has been further substantiated by bioinformatic and pedigree-based studies from the Netherlands and the UK, that found *C9orf72* expansions in around 0·12% of the general population, corresponding to a carrier rate of 1 in 839 individuals.[24]

Most expansion carriers clinically present with ALS or frontotemporal dementia, as diagnosed by established criteria;[25,26] however, other neurological diseases have also been associated with these mutations, such as Alzheimer's disease and Huntington's disease-like syndrome.[27] But it should be noted that a neuropathological confirmation of clinical diagnoses other than ALS and frontotemporal dementia are often absent. Intriguingly, in a study of patients in Ireland,[28] *C9orf72* kindreds appeared to be discordant, with some family members developing ALS without apparently carrying the repeat expansion. Although further investigations are warranted, this observation could be explained by the presence of somatic expansions only in the brain. It is also reasonable to hypothesise that additional genetic or environmental factors contribute to the risk of the disease in these families of mutation carriers. The possibility of additional genetic or environmental factors is in line with higher hazard ratios of schizophrenia and suicide among first degree relatives of *C9orf72* expansion carriers, compared with population-based controls,[29] and the observation of a discernible cognitive endophenotype in blood-relatives of expansion carriers that is not present in relatives of patients with non-familial ALS.[30] In fact, emerging evidence suggests an oligogenic component to ALS.[31,32] For instance, intermediate *ATXN2* repeat expansions can be detected in some *C9orf72* expansion carriers, and are associated with having a higher risk of developing ALS than those who do not carry a repeat expansion within *ATXN2*,[33] whereas variants in *TMEM106B* appear to protect against developing frontotemporal dementia in individuals who carry the *C9orf72* repeat expansion.[34,35] Taken together, these data suggest that phenotypes can be clinically diverse and genetically more complex than previously envisaged. Future in-depth studies of *C9orf72* expansion carriers, ideally including large pedigrees, such as those collected as part of the Frontotemporal Dementia Prevention initiative[36] and the Pre-symptomatic Familial ALS Study,[37] should aid in identifying additional phenotypic modifiers and providing guidance for genetic counselling. These longitudinal cohort studies are also crucial for our understanding of the natural history of *C9orf72* disease.

### *Panel 1*: Glossary of terms

*Short-read sequencing*: sequencing method that covers short DNA fragments, often 100 to 300 base pairs

*Long-read sequencing*: sequencing method that spans long DNA fragments, generally 10 to 20 kilobases

*Penetrance*: the likelihood of a mutation carrier to develop symptoms characteristic for a pathogenic gene variant

*Repetitive or repeat RNAs*: RNAs transcribed from both sense and antisense strands that contain an expanded repeat

*Dipeptide repeat proteins*: proteins generated from unconventional translation of RNAs that harbour an expanded repeat

*Somatic expansions*: expanded DNA repeats that, after birth, become longer in cells over time

*Cognitive endophenotype*: quantifiable, heritable trait that reflects underlying cognitive processes closer to the biological foundations of the disease than its observable symptoms

*Oligogenic component*: refers to several genetic variants acting together in developing a disease or modifying its onset or presentation

*Phenoconversion*: in a mutation carrier, the shift from a healthy to a clinical stage

## Early and distinct brain changes

The *C9orf72* repeat expansion is unique among the mutations associated with ALS in its ability to lead to distinct clinical phenotypes (from pure frontotemporal dementia to pure ALS, and other clinical syndromes resembling Huntington's disease). Indeed, some patients present with mixed phenotypes and do not meet either ALS or frontotemporal dementia diagnostic criteria.[38] At a group level, patients with *C9orf72*-ALS appear to be clinically distinct from those with apparently non-familial ALS (table 1). Neuroimaging studies of patients with *C9orf72*-ALS reveal involvement of the motor cortex, along with extra-motor and deep grey matter changes, including the thalamus, that differs from other forms of ALS.[39,40] However, within a clinical setting, it is not possible to reliably distinguish individuals carrying the *C9orf72* repeat expansion from those who do not carry the expansion. At the group level, clinical features that distinguish *C9orf72*-FTD from other forms of apparently non-familial FTD include a higher frequency of delusions, greater impairment of working memory, and milder eating dysregulation.[41,42] Neuroimaging studies have reported a greater degree of thalamic atrophy in

For more on the **Frontotemporal Dementia Prevention Initiative** see https://thefpi.org/

For more on the **Pre-symptomatic Familial ALS Study** see https://als-research.org/research-study/pre-fals-pre-symptomatic-familial-als-study/

| | C9orf72-ALS | Non-familial ALS* |
|---|---|---|
| Median age of onset[134] | 59·6 years (95% CI 40·3–76·9) | 64·5 years (95% CI 36·5–82·2) |
| Concomitant frontotemporal dementia[135] | About 50% | About 15% |
| Family history of ALS or frontotemporal dementia[135] | Frequent | Occasional |
| Mean survival | 35·5 months (95% CI 33·8–37·2) | 42·2 months (95% CI 41·4–42·9) |
| Intensive physical activity as a risk factor[137] | Likely | Inconclusive evidence |
| Pre-morbid cognitive endophenotype[33] | Frequent | Rare |
| Endophenotype in first degree relatives[26] | Frequent | Rare |

ALS=amyotrophic lateral sclerosis. *C9orf72*-ALS=*C9orf72*-associated ALS. *Absence of known mutation.

***Table 1:*** **Comparison of clinical features between patients with *C9orf72*-ALS or non-familial ALS**

*C9orf72* mutation carriers, either with ALS or frontotemporal dementia, than in patients with these diseases who are non-carriers.[41]

Although *C9orf72*-FTD typically presents in late adulthood, reduced verbal fluency has been reported in mutation carriers before disease onset[43] and structural brain changes, including reduced cortical gyrification[44] and changes in the thalamus and posterior cortical areas that occurred up to 20 years before the projected disease onset.[45] These findings suggest that the *C9orf72* mutation might have effects early in brain development and implies that clinical phenotypes are a late manifestation of a lifelong process by which the mutation might play an important adverse role in early neurodevelopment. Indeed, during life, other genetic or epigenetic factors, including environmental exposures, can interact with pre-existing cellular and network vulnerabilities. Clinical manifestation would then occur when compensatory mechanisms that attenuate risk are overwhelmed, leading to a clinical phenotype and a progressive process of neuronal loss and network disintegration. Understanding the connections between genetic risk, compensatory mechanisms, clinical phenotype, and disease progression is fundamental for the successful development and administration of therapeutics, and for disease management. If *C9orf72* repeat expansions affect early neurodevelopmental processes, understanding the factors that lead to the tipping points that drive clinical manifestations will be essential for early therapeutic intervention.

## Neuropathology

As in patients with apparently non-familial ALS, TDP-43 aggregates are also the neuropathological hallmark for patients with *C9orf72*-ALS. This primary neuropathological hallmark is the result of the mis-localisation of the TAR DNA and RNA binding protein TDP-43 in the CNS, including in the spinal cord.[46] This neuropathological hallmark is also shared by most cases of apparently non-familial ALS and by about 45% of cases with apparently non-familial frontotemporal dementia, and most other genetic forms of ALS, except those caused by *FUS* or *SOD1* mutations. TDP-43 pathology is found predominantly in neurons, but also in glia. The TDP-43 neuropathology type A corresponds to abundant neuronal cytoplasmic inclusions and short dystrophic neurites in cortical layer II, with occasional intranuclear inclusions, whereas type B corresponds predominantly to diffuse neuronal cytoplasmic inclusions across all cortical layers, with few dystrophic neurites. Unique to *C9orf72*-FTD cases, they frequently have a combination of both TDP-43 type A and B neuropathology.[47]

Additional neuropathological hallmarks that are unique to *C9orf72*-ALS and *C9orf72*-FTD include accumulation of repeat RNA transcribed from both sense and antisense strands, and dipeptide repeat proteins that result from unconventional translation of repeat RNA. Both sense and antisense repeat RNA accumulate into foci that are predominantly nuclear, but also occur in the cytoplasm, and can be found in the CNS and periphery (figure 2).[48–51] Antisense foci are more numerous in neurons than sense foci, and often have a peri-nucleolar localisation.[52] Consistent with their lack of specificity to brain regions of clinical relevance, studies that have tried to correlate the location of RNA foci with clinical features do not support a clear pathogenic role.[53,54] However, multiple studies have shown an association of antisense RNA foci with TDP-43 pathology,[52,55] and an enhanced RNA foci detection method has shown an association of TDP-43 pathology with sense foci specifically in spinal motor neurons.[53]

The translation of the repeat RNA can occur in all reading frames from both sense and antisense RNA strands, resulting in the production of five different dipeptide repeat proteins: polyGA, polyGR, polyGP, polyPA, and polyPR.[48] All five dipeptide repeat proteins are detected neuropathologically (figure 2), with the sense-encoded polyGA being the most abundant in the CNS, followed by the sense-encoded polyGP and polyGR;[49,50] polyGA and polyGP have also been found in skeletal muscle of patients with *C9orf72*-ALS.[51] Similar to TDP-43, inclusions of dipeptide repeat proteins occur predominantly in the cytoplasm of neurons, but also occasionally as small dot-like nuclear inclusions and in dystrophic neurites. While aggregates of dipeptide repeat proteins are specific to *C9orf72* expansion carriers, their importance in pathogenesis is uncertain since they are variably present and do not correlate with disease-relevant CNS areas, unlike TDP-43 pathology.[49,50] For instance, inclusions of dipeptide repeat proteins can be detected in the cerebellum and occipital cortex, even in cases with minimal neurodegeneration and TDP-43 pathology.[46,50] It has been postulated that dipeptide repeat proteins might be early initiators of disease, which could explain the lack of correlation of the location of their inclusions with clinical symptoms.[56] Some studies have reported an association of

polyGR with TDP-43 pathology[57] and regions of neurodegeneration.[58]

## Pathogenic mechanisms

### Repeat DNA

*C9orf72* repeat expansions can exert pathogenicity through both loss and gain of function mechanisms. Sense and antisense repeat RNAs bind specific RNA-binding proteins and sequester them into foci.[59] In tissue from patients with the mutation, transcriptomic signatures are consistent with the loss of these proteins.[60] Early work largely focused on the effects of the sense GGGGCC repeat RNAs, but a growing body of evidence now shows the effects of the antisense repeat RNA. This evidence is particularly relevant given the failure of sense repeat-targeting antisense oligonucleotides in clinical trials, which suggests that antisense repeat RNA or dipeptide repeat proteins might play an important role.[61] Sequestration of the phenylalanine-tRNA synthetase subunit-α by antisense repeat RNA leads to reductions in the incorporation of the amino acid phenylalanine during translation, which can compromise neuronal function.[62] In induced pluripotent stem-cell neurons (iPSN) of patients with *C9orf72*-ALS or *C9orf72*-FTD, the turnover of nuclear pore complex components is impaired and has been linked to RNA toxicity.[63] Of interest, targeting antisense (but not sense) repeat RNA in iPSNs alleviates gene expression and splicing alterations associated with loss of nuclear TDP-43,[64] in line with the selective association of antisense foci with TDP-43 pathology in human tissue.[52] Antisense repeat RNAs can also activate the integrated stress response and, in a zebrafish experimental model, the inhibition of the integrated stress component protein kinase R alleviates neurotoxicity.[65]

### Dipeptide repeat proteins

In several experimental models, all five dipeptide repeat proteins produced from the *C9orf72* repeat expansion have been shown to exert neurotoxicity, with the arginine-rich polyGR and polyPR being the most toxic, followed by polyGA. However, polyPA and polyGP are non-toxic in most studies.[66,67]

According to evidence from in vitro studies, the positively charged arginine-rich dipeptide repeat proteins (polyGR and polyPR) have an avidity for membrane-less organelles, such as RNA granules, and also for the nucleolus and the nuclear pore, and alter the function of these organelles by disrupting liquid–liquid phase separation behaviour,[68–70] a mechanism by which proteins and RNA undergo multivalent interactions forming dynamic liquid condensates without the requirement for membrane-bound vesicle formation. Due to the diversity of the affected cellular systems, the effects of arginine-rich dipeptide repeat proteins range widely, affecting genomic stability, RNA splicing and transport, translation, and nucleus-to-cytoplasm transport.[69,71] In addition, there is evidence that arginine-rich dipeptide repeat proteins also contribute to TDP-43 mis-localisation via processes such as altered nucleus-to-cytoplasm transport (by sequestration of transport factors),[72–75] aberrant nucleolar function,[68,70] and the nucleation of TDP-43 aggregation;[76] in agreement with these findings, TDP-43 proteinopathy develops in several experimental models of arginine-rich dipeptide repeat proteins.[67,72] In neurons, arginine-rich dipeptide repeat proteins can also directly bind to microtubules and impair transport,[77] to ribosomes and impair translation,[78]

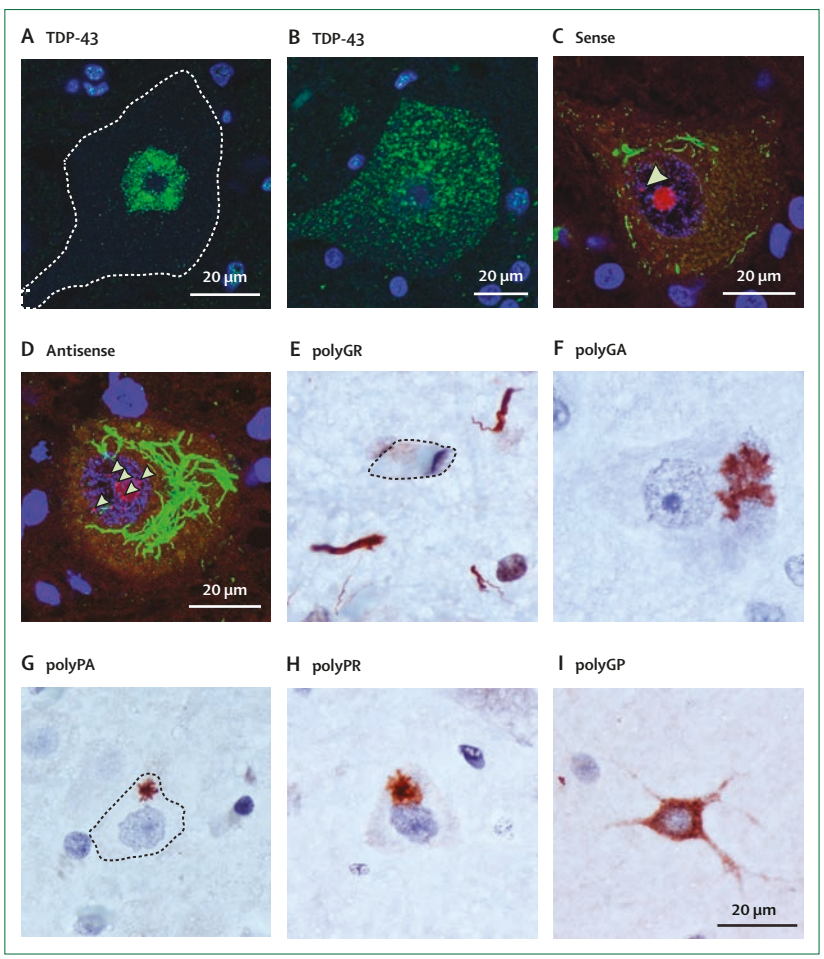


***Figure 2: C9orf72*-associated neuropathology**
(A) Immunofluorescence in a healthy motor neuron detecting nuclear TDP-43 (antibody to TDP-43 in green and DAPI nuclear staining in blue). (B) Motor neuron from a patient with *C9orf72*-ALS with loss of nuclear TDP-43 and aggregation in the cytoplasm, detected by immunofluorescence (antibody to TDP-43 in green and DAPI in blue). (C) Sense-RNA foci in the nucleus of a motor neuron with loss of nuclear TDP-43 but with cytoplasmic aggregates, detected by co-immunofluorescence in situ hybridisation (antibody to TDP-43 in green, probes complementary to the sense RNA foci containing GGGGCC-repeats in red, and DAPI in blue). The red signal in the nucleolus is non-specific. (D) Multiple antisense-RNA foci in the nucleus of a motor neuron with loss of nuclear TDP-43 but with cytoplasmic aggregates, detected by co-immunofluorescence-in situ hybridisation (antibody to TDP-43 in green, probes complementary to the antisense RNA foci containing GGCCCC-repeats in red, and DAPI in blue). The signal around the nucleolus is only detected with antisense foci. (E–I) Neurons from the cortex of patients with *C9orf72*-ALS showing aggregated dipeptide repeat proteins encoded by the GGGGCC repeat expansion in *C9orf72*. Dipeptide repeat proteins are visualised by immunohistochemistry with antibodies specific to each of the of them. G=glycine; R=arginine; A=alanine; P=proline. Reproduced with permission from *Acta Neuropathologica*.[57]

and to mitochondrial components, with resultant DNA damage and glucose hypometabolism.[79] Furthermore, in knock-in mouse models, the expression of arginine-rich dipeptide repeat proteins using the endogenous mouse C9orf72 promoter induces increased levels of extracellular matrix proteins in the spinal cord, which provide protection against neurodegeneration.[80]

PolyGA is the most aggregation-prone dipeptide repeat protein and it can sequester other dipeptide repeat proteins and cellular factors, including chaperones[81] and proteasome components, which could further promote protein aggregation; experimental models of polyGA also develop TDP-43 proteinopathy. Notably, and similar to other misfolded proteins involved in neurodegeneration, there is evidence for cell-to-cell transmission of dipeptide repeat proteins.[82] Taken together, findings suggest that widespread changes occur when dipeptide repeat protein are expressed in experimental models, thus, a major challenge is determining which dipeptide repeat proteins are the most relevant as therapeutic targets.

## C9orf72 loss of function

The presence of the repeat expansion in the promoter region of *C9orf72* V2 (figure 1) induces hypermethylation and reduced expression of *C9orf72*, causing loss-of-function. The C9orf72 protein has homology to the differentially expressed in normal cells and neoplasia (DENN) family of proteins, which primarily regulate Rab proteins and have roles in membrane trafficking. C9orf72 can affect multiple pathways in neurons and glia, ranging from autophagy and lysosomal homeostasis to actin dynamics, nucleocytoplasmic transport, lipid metabolism, and the regulation of postsynaptic receptor recycling at the synapse.[83] The C9orf72 protein is ubiquitously expressed in most tissues throughout the body but its highest concentrations are in the brain and spinal cord, with enrichment in neurons and myeloid-lineage cells; which is consistent with early findings showing that loss of C9orf72 in mouse models caused primarily an immune system phenotype.[67] Indeed, loss of C9orf72 in myeloid cells impairs the degradation of a key protein in the innate immune response, stimulator of interferon genes (STING), resulting in a hyperactive type I interferon response.[84] In *C9orf72* knockout mice, peripheral inflammation can be ameliorated by immune-stimulating gut bacteria[85] and interleukin-17A reduction,[86] which might be strategies to modulate C9orf72-related immune pathways. In mice, the partial knockdown of *C9orf72* causes apathy and social behaviour dysfunction, mild motor impairment, and importantly, neuronal TDP-43 aggregates in old mice. These TDP-43 aggregates indicate a potential interaction of aging and C9orf72 loss-of-function in causing TDP-43 pathology.[87] Additionally, loss of C9orf72 can exacerbate the toxicity of dipeptide repeat proteins, likely due to the disruption of autophagy.[88,89] These findings provide evidence that *C9orf72* haploinsufficiency can affect key pathways in both neurons and glia in the CNS and in the peripheral immune system, with the weight of evidence indicating a direct contribution to neuronal vulnerability via both cell-intrinsic and non-cell autonomous mechanisms. Consequently, therapeutic strategies should avoid further reducing C9orf72 expression. Rather, enhancing C9orf72 expression, or modulating its immune functions, could provide protection from the toxicity induced by repeat expansions.

## DNA repeats

Unexpectedly, new evidence suggests an additional pathogenic role of the *C9orf72* repeat expansion. The *C9orf72* repeat expansion DNA binds to and causes nuclear accumulation of the DNA-binding protein DAXX, leading to alterations in epigenetics and chromatin structure. Furthermore, in neurons, DAXX nuclear accumulation suppresses *C9orf72* expression, preventing stress-induced upregulation of *C9orf72*, which increases neuronal vulnerability and can be rescued by decreasing DAXX concentrations.[90] This finding is in agreement with recent transcriptomic studies showing altered chromatin and epigenetic signatures in post-mortem brain and iPSN from patients with *C9orf72*-ALS or *C9orf72*-FTD.[91,92] A new study also suggests that the repeat expansion can cause chromosomal instability, which could then trigger DNA damage or an immune stimulation response, thus providing further pathways by which the DNA repeats could contribute to pathogenesis.[93]

## Convergent mechanisms

A reproducible hallmark of iPSNs derived from *C9orf72* mutation carriers is their sensitivity to glutamate excitotoxicity, which can result from either the loss or gain of function caused by the mutation.[94–96] Altered nucleocytoplasmic transport,[97] lipid metabolism,[98,99] and activation of the innate immune system STING signalling pathway[84,100] have also been reported in both loss-of-function and gain-of-function contexts, all of which compromise neuronal health. Lastly, as TDP-43 proteinopathy is a neuropathological hallmark in patients that carry the mutation and has been observed in both loss and gain of function models, TDP-43 associated pathomechanisms might play a key convergent role.

How *C9orf72* pathogenic mechanisms (figure 3) lead to the diverse clinical presentations in the ALS–frontotemporal dementia spectrum is unclear. The widespread distribution of repeat RNA and dipeptide repeat protein pathologies suggests an intrinsic neuronal vulnerability in mutation carriers to developing TDP-43 pathology. This neuronal vulnerability might arise from oligogenicity, somatic mosaicism, or other yet undefined intrinsic or environmental factors.

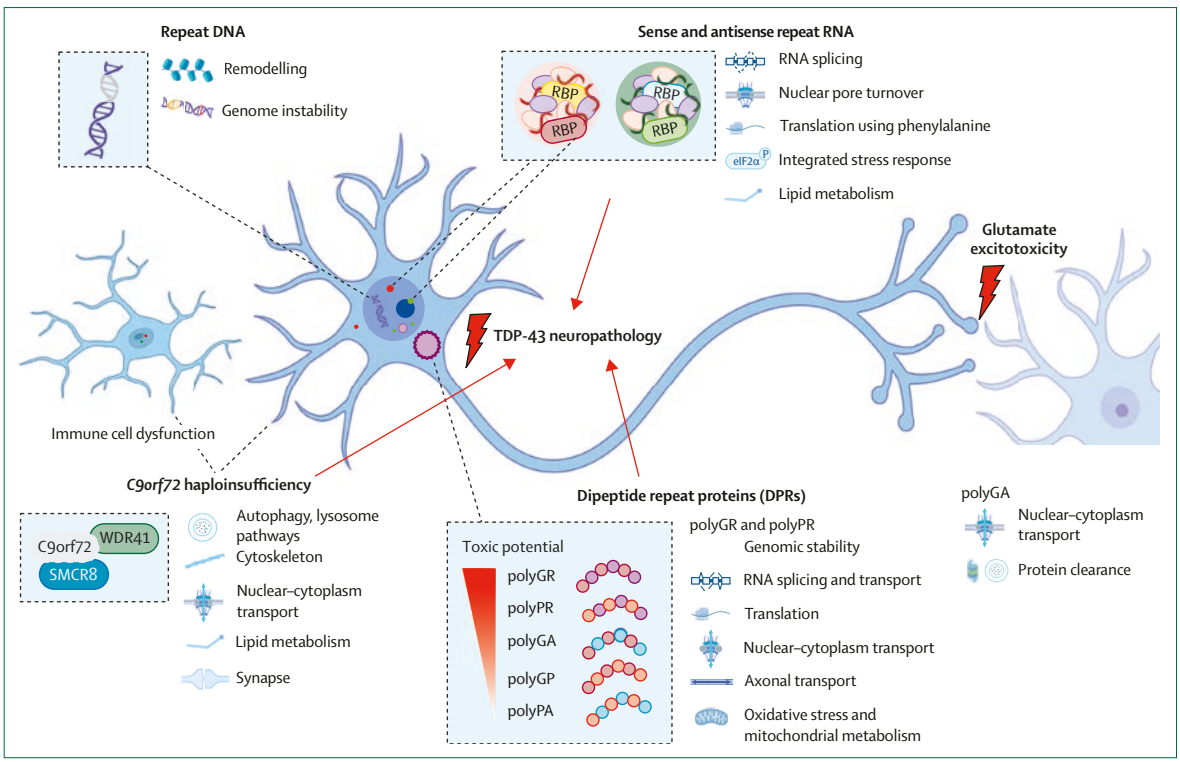


***Figure 3:*** **Pathogenic mechanisms of the *C9orf72* repeat expansion**
The *C9orf72* repeat expansion can exert pathogenesis by gain of functions from the repeat DNA, sense and antisense repeat RNA, and dipeptide repeat proteins, and loss of function of the C9orf72 protein. Hence, there is a diversity of cellular pathways affected. The repeat DNA sequesters DAXX, leading to chromatin remodelling and transcriptional changes, including reduced C9orf72 expression. Sense and antisense repeat RNA form foci that sequester RNA-binding proteins and affect RNA splicing, nuclear pore integrity, translation using phenylalanine, and the integrated stress response. Dipeptide repeat proteins form inclusions. The most toxic polyGR or polyPR disrupt liquid–liquid phase separation membrane-less organelles (eg, nucleolus, RNA granules, and nuclear pore) and bind microtubules and mitochondria; these effects impair genomic stability, RNA splicing and transport, translation, nucleus–cytoplasm and neuritic transport, and oxidative stress. PolyGA is the most aggregation prone dipeptide, and can sequester other dipeptide repeat proteins, nucleus–cytoplasm transport and proteosome components, and chaperones to disrupt nucleus–cytoplasm transport and protein clearance. *C9orf72* haploinsufficiency causes loss of the C9orf72 protein, affecting its role in membrane trafficking, which in turn affects autophagy, lysosome pathways, the cytoskeleton, nuclear–cytoplasm transport, lipid metabolism, and synapse function. Disruption of these processes can cause immune cell dysfunction (interferon response) and exacerbate toxicity. A=alanine. DPR=dipeptide repeat protein. G=glycine. P=proline. R=arginine.

## Fluid biomarkers

Biomarkers that facilitate an early diagnosis, inform prognosis, predict phenoconversion, or monitor responses to therapeutic interventions are urgently needed to improve patient care and therapeutics. In these regards, neurofilament and dipeptide repeat protein proteins might be useful, and new endeavours in TDP-43 biomarker discovery are well poised to further expand the biomarker arsenal.

### Neurofilament

Neurofilaments (intermediate filaments expressed exclusively in neurons) are composed of three subunits, each defined by their molecular weight: neurofilament light (NfL; around 60 kDa), neurofilament medium (around 100 kDa), and neurofilament heavy (around 110 kDa).[101] Neurofilaments, and NfL in particular, are established markers of neuronal injury with prognostic utility for patients with ALS and frontotemporal dementia, including those with a *C9orf72* repeat expansion.[102–104] Accordingly, NfL concentrations in blood or CSF could improve clinical trial design by enabling the stratification of participants with fast or slow disease progression. NfL might also serve as a response biomarker; for instance, two clinical trials (NCT04288856 and NCT04931862) testing distinct investigational antisense oligonucleotides that target sense GGGGCC repeat transcripts failed to show clinical benefit when compared with placebo. In fact, participants receiving antisense oligonucleotides had higher CSF and blood NfL concentrations than those receiving placebo, in line with the intervention failing to show clinical benefit.[105] In tandem with neuropsychological test scores, advanced neurophysiology, and neuroimaging biomarkers, increases in NfL might also inform about impending symptom onset in pre-symptomatic mutation carriers,[104,106–108] which would allow for their recruitment in prevention or early treatment trials. This approach is being evaluated for pre-symptomatic carriers of *SOD1* variants associated with rapid disease progression in a

trial (NCT04856982) testing whether a *SOD1*-targeting antisense oligonucleotide delays the clinical manifestation of ALS.[109] However, for pre-symptomatic *C9orf72* repeat expansion carriers, longitudinal data spanning phenoconversion are scarce, rendering it difficult to establish the period between rising NfL concentrations and subsequent symptom onset. Although disease progression models suggest that NfL is elevated 1–5 years before estimated onset,[110] further work is required before prevention trials can be designed for pre-symptomatic *C9orf72* repeat expansion carriers. Preliminary studies suggest that microglia play a role in clearing NfL and that some drugs, such as minocycline, inhibit this clearance, thereby eliciting an increase in NfL in the absence of neurodegeneration.[111] Consequently, minocycline use could confound the interpretation of NfL measures.

### Dipeptide repeat proteins

The discovery of dipeptide repeat protein in *C9orf72* repeat expansion carriers and the ensuing studies in experimental models showing that these proteins are toxic have led investigators to assess their prognostic utility.[102,112–115] Contrary to expectations that the abundance of dipeptide repeat protein would track with clinical severity, CSF concentrations of dipeptide repeat protein do not associate with age at disease onset, Amyotrophic Lateral Sclerosis Functional Rating Scale score, survival after symptom onset, disease (ALS or frontotemporal dementia), or NfL concentrations.[102,112–115] Their poor performance as prognostic markers notwithstanding, dipeptide repeat protein might be useful as pharmacodynamic biomarkers in experimental models and clinical trials. Indeed, investigational antisense oligonucleotides targeting the repeat expansion decrease CSF concentrations of polyGP, polyGA, and polyGR,[105,113,116,117] showing target engagement but, alas, not clinical benefit.

### Biomarkers of TDP-43 pathology

TDP-43 neuropathology, a hallmark feature of *C9orf72*-ALS and *C9orf72*-FTD, is characterised by the mislocalisation of TDP-43 to the cytoplasm, where it forms aggregates, and the depletion of TDP-43 from the nucleus resulting in its loss of function. One such function of TDP-43 is to repress the inclusion of cryptic exons during RNA splicing. Because the failure of TDP-43 to do so can result in the production of cryptic exon-encoded peptides, such peptides are being investigated as biomarkers of TDP-43 pathology or loss of function.[118–120] For instance, a cryptic protein derived from the gene hepatoma-derived growth factor-like protein 2 (HDGFL2), a histone-binding protein expressed in the brain that regulates chromatin accessibility and assists in DNA damage repair, can be detected by use of an immunoassay in CSF and blood samples from patients with a *C9orf72* repeat expansion,[119] and its expression in post-mortem brain, also measured by use of an immunoassay, is positively associated with phosphorylated TDP-43 levels in the brain.[120] Emerging methods to measure TDP-43 in plasma extracellular vesicles[121] and to detect TDP-43 aggregates in CSF using real-time quaking-induced conversion seeding assays[122] might also speed up the development of TDF-43-based prognostic, predictive, or pharmacodynamic markers.

## Therapeutic approaches

Several therapeutic strategies have been developed over the past 10 years to tackle the pathological effects of *C9orf72* repeat expansions. These strategies include removing the genomic repeat expansion, reducing the expression of *C9orf72* repeat transcripts and dipeptide repeat proteins , and manipulating modifiers of TDP-43 pathology.

### RNA and CRISPR-Cas based approaches

Antisense oligonucleotide therapies were rapidly developed to target sense repeat transcripts for degradation.[123,124] These antisense oligonucleotides targeting sense *C9orf72* repeat transcripts led to promising neuroprotective outcomes in experimental models;[123,124] however, they failed in clinical trials,[117,149] despite target engagement and reduced concentrations of polyGP and polyGA in the CSF of patients. A decrease in polyGP and polyGA should not be interpreted as a reduction in all dipeptide repeat proteins, particularly those translated from antisense *C9orf72* repeat transcripts. Measuring antisense specific dipeptide repeat proteins in samples from participants in the trials would provide a better understanding of disease mechanisms. On the other hand, antisense oligonucleotides targeting sense *C9orf72* repeat transcripts might have led to some off-target degradation of non-expanded transcripts encoding the C9orf72 protein, as reported in some initial mouse studies. Either way, the failure of these trials brings focus on developing new drugs that can target gain-of-function and combinatory mechanisms.

Gene editing with CRISPR-Cas systems has been successfully evaluated in patient-derived neurons and mouse brains. These systems were engineered to (1) delete the genomic repeat expansions[125] or the promoter in exon 1a and the expression of repeat-containing V1 or V3 isoforms,[126] (2) impede transcription of *C9orf72* sense transcripts using deactivated Cas9,[127] or (3) induce the degradation of both sense and antisense repeat transcripts using RNA-targeting Cas enzymes.[128,129]

A ribonuclease-targeting chimera (ie, RiboTAC), that directly binds GGGGCC hexanucleotides in sense *C9orf72* repeat RNAs and recruits RNase L, induced the degradation of sense repeat transcripts in patient-derived neurons and mouse brains.[130] Further research of these

| | Drug | Target | Trial number | Trial phase | Participants | Outcomes |
|---|---|---|---|---|---|---|
| BIIB078 | ASO | Degradation of *C9orf72* pre-mRNA variants V1 and V3, with some off-target effects in the V2 isoform | NCT03626012 | 1 | 106 patients with *C9orf72*-ALS | Halted in March, 2022 (no clinical benefit and functional decline at highest dose [A: correct?])[106] |
| WVE-004 | Stereopure ASO | Degradation of V3 preferentially and V1 isoforms, without targeting V2 | NCT04931862 | 1/2 | 35 patients with *C9orf72*-ALS or *C9orf72*-FTD | Halted in May, 2023 (no clinical benefit reported)[118] |
| Metformin | Repurposed small molecule | Inhibition of RAN translation | NCT04220021 | 2 | 18 patients with *C9orf72*-ALS | The study is ongoing |
| TPN-101 | Repurposed small molecule | LINE-1 retrotransposon inhibitor | NCT04993755 | 2a | 42 patients with *C9orf72*-ALS or *C9orf72*-FTD | Clinical benefits reported [A: please specify outcome measures] |
| Apilimod | Repurposed small molecule | PIKFYVE kinase inhibitor that stimulates clearance of aggregated proteins via exocytosis | NCT05163886 | 2a | 14 patients with *C9orf72*-ALS | Safety and biomarker endpoints met[141] |
| BIIB100 | Small molecule inhibitor (KPT-350, Karyopharm Therapeutics) | XPO1 inhibitor modulating the karyopherin-dependent nuclear export of proteins and some non-coding RNAs and toxicity of arginine-rich dipeptide repeat proteins | NCT03945279 | 1 | 49 patients with ALS | Halted in June, 2022; no clinical benefit reported |
| Latozinemab | Monoclonal antibody | Targeting sortilin, to inhibit lysosomal degradation of GRN and to increase GRN levels | NCT03987295 | 2 | 16 patients with *C9orf72*-FTD or frontotemporal dementia due to *GRN* mutations | Halted for C9orf72-FTD (no clinical benefit); ongoing phase 3 for carriers of GRN mutations [A: please supply NCT number for this phase 3] |
| BIIB105 | ASO | Degradation of ATXN2 mRNA to reduce ATXN2 protein levels and target persistent stress granules and protein aggregates | NCT04494256 | 1/2 | 99 patients with ALS with or without intermediate length CAG repeat expansions in *ATXN2* | Halted in May, 2024 (no clinical benefit reported) |
| Lithium carbonate | Repurposed inorganic salt | Promotion of synaptogenesis and autophagy | NCT06008249 | 3 | 171 patients with ALS with *UNC13A* mutations (homozygous for the C-allele at single nucleotide polymorphism rs12608932) | The study is ongoing |
| QRL-201 | ASO | Restauration of STMN2 protein expression via splicing modulation | NCT05633459 | 1 | 64 patients with ALS (excluding patients with *SOD1* or *FUS* mutations) | The study is ongoing |

ALS=amyotrophic lateral sclerosis. ASO=antisense oligonucleotide. ATXN2=ataxin 2. *C9orf72*-ALS=*C9orf72*-associated ALS. *C9orf72*-FTD=*C9orf72*-associated FTD. DPR=dipeptide repeat protein. FTD=frontotemporal dementia. GRN=progranulin. RAN=Repeat-associated non-AUG. STMN2=stathnin 2. XPO1=exportin 1.

***Table 2:*** **Clinical developments for patients with *C9orf72*-ALS or *C9orf72*-FTD**

gene editing technologies will be necessary to safely bring this new type of drugs into the clinic.

### Targeting dipeptide repeat proteins

The inhibition of the nuclear export of both sense and antisense *C9orf72* repeat transcripts by partial depletion of serine–arginine-rich splicing factor 1 (SRSF1)[131] or administration of a SRSF1-inhibitory cell permeable peptide[132] led to reduced expression of dipeptide repeat proteins and neuroprotection in patient-derived motor neurons and in fruit flies. The partial depletion of SRSF1 promotes neuronal survival by the activation of homeostasis pathways in neurons.[133] Metformin, a repurposed drug used to treat patientes with type 2 diabetes, also inhibits protein kinase R, reducing the translation of dipeptide repeat proteins and rescuing neurodegeneration-associated deficits in mice,

***Panel 2:*** **Research priorities to accelerate therapeutics to prevent or delay *C9orf72*-associated amyotrophic lateral sclerosis**

- Understand the genetic and environmental factors that affect the penetrance of the *C9orf72* repeat expansion
- Develop TDP-43 neuroimaging and blood biomarkers
- Develop methods to measure antisense repeat-derived RNAs
- Describe the downstream pathways of *C9orf72* mutations in both neurons and immune cells
- Implement clinical trial platforms for *C9orf72* mutation carriers capable of testing multiple therapies simultaneously, by comparison with a single placebo group

including gait alterations and anxiety-like phenotypes.[134] Metformin is now being evaluated in a phase 2 clinical trial in patients with *C9orf72*-ALS or *C9orf72*-FTD (NCT04220021).

Clearance of dipeptide repeat proteins has been also achieved via (1) intraperitoneal administration of human anti-polyGA neutralising antibodies in mice, to reduce expression and impair cell-to-cell transmission of polyGA,[135–137] (2) overexpression of heat shock protein family B member 8 (HSPB8) in motor neuron-like NSC34 cells to promote autophagy-mediated disposal of all dipeptide repeat proteins,[138] and (3) treatment with apilimod, a repurposed PIKFYVE kinase inhibitor that activates the exocytosis of aggregation-prone proteins[139] and was recently shown to lower CSF polyGP concentrations in patients with *C9orf72*-ALS in a phase 2a clinical trial.[140]

### Targeting modifiers of TDP-43 pathology

Reduced TDP-43 pathology and less severe phenotypes were observed in mouse models of TDP-43 proteinopathy after the administration of anti-TDP-43 monoclonal antibodies[141,142] or following depletion of *ATXN2* (a gene that codifies for an RNA-binding protein) by use of antisense oligonucleotides[143] or the CRISPR-Cas13 system.[144] However, translation into the clinic might be restricted, since experimental models in mice do not recapitulate TDP-43 proteinopathy in human beings, and an *ATXN2*-targeting antisense oligonucleotide trial in patients with ALS with or without intermediate CAG repeat expansions in *ATXN2* did not show any clinical benefits.[145] Another proposed therapeutic strategy has been to target the effects of TDP-43 nuclear loss-of-function. This strategy involves blocking the cryptic mis-splicing of *STMN2* to restore its expression in neurons using an antisense oligonucleotide or an RNA-targeting Cas system.[146] However, restoring the expression of *STMN2* might not be sufficient, since the loss of function of TDP-43 leads to dozens of cryptic mis-splicing events, including in the gene *UNC13A*, which is also associated with ALS and frontotemporal dementia.[147,148]

### Progress in clinical trials

To date, early-phase trials testing therapeutic approaches have not shown efficacy, highlighting the need for a better understanding of pathological mechanisms. Previous and ongoing studies are listed in table 2. Lastly, given the heterogeneity in clinical presentation of patients with *C9orf72*-ALS or *C9orf72*-FTD, specific outcome measures of motor, cognition, and behavioural testing could be considered in new clinical trial designs.[150,151] Additional considerations that are crucial for future success of clinical trials involve the selection of the most appropriate route of drug delivery and the identification of diagnostic biomarkers.[38]

#### Search strategy and selection criteria

References in this Review were identified from searches of PubMed with the term "C9orf72". The final reference list was generated based on their relevance to the topics covered in this Review, with particular emphasis placed on papers published in the past 5 years. Our search was conducted from 2011 until October, 2024.

## Conclusions and future directions

Since the discovery of the repeat expansion about 14 years ago, there have been major advances in the clinical and molecular understanding of *C9orf72*-related diseases. The complexity of this mutation is now clear, with both loss-of-function and gain-of-function effects, and pathogenic consequences via a wide range of cellular processes in neurons and glia, both in the CNS and periphery. Notwithstanding, there are clear points of convergence, such as TDP-43 proteinopathy in most patients. The first wave of therapeutics has shown that targeting sense *C9orf72* repeat RNA alone is insufficient to alleviate neurodegeneration, highlighting the urgent need to identify and target also other pathological drivers of clinical onset and disease progression. The development of biomarkers would improve future clinical trials by allowing recruitment of participants at early disease stages and could facilitate stratification and monitoring of target engagement (panel 2). This is an exciting time in *C9orf72* research and there is growing optimism that effective therapeutics can be developed to improve the lives of people with these severe conditions.

#### Contributors

RR and AMI conceptualised and supervised the manuscript. RR and MvB wrote the sections on the GGGGCC repeat expansion and genetic diagnosis and clinical correlations, and created figure 1. OH contributed to the section on genetic diagnosis and clinical correlations and created table 1. OH, RR, and MvB wrote the section on key clinical considerations and created table 1. JR wrote the section on neuropathology and created figure 2. SM and AMI wrote the section on pathogenic mechanisms and created figure 3. TFG wrote the sections on fluid biomarkers. GMH wrote the section on therapeutic approaches and created table 2. All authors contributed to the writing and editing of the Review, with SM leading the integration of the individual contributions.

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SM received payments to her institution from UK Dementia Research Institute, the Motor Neurone Disease (MND) Association, the Packard Centre for ALS Research, Van Geest Neurosciences Donation, Alzheimer's Research UK, and ONO Pharmaceuticals; and participates in Discovery Network Advisory Board: My Name5 Doddie. TFG received funding from the National Institutes of Health, National Institute on Aging (NIA), and the National Institute of Neurological Disorders and Stroke (NINDS; P30 AG062677, U19 AG063911, P01 NS084974, R01 NS117461, and R01 NS121125); and received inventor intellectual property royalties from Ionis Pharmaceuticals, Takeda, Biogen, and Jackson Laboratory, and the Target ALS for her involvement in the development of the *C9orf72* repeat expansion construct and an *AAV-C9orf72* repeat expansion mouse model. GMH received grants from the Medical Research Council (MRC; grants MR/W00416X/1 and MR/Z506229/1), the Biotechnology and Biological Sciences Research Council (grant BB/S005277/1), and the LifeArc Philanthropic Fund MND Association grant 878-791; and is the Founding Director of Crucible Therapeutics and

the primary inventor of granted and pending patents related to the use of SRSF1 inhibitors to treat neurological disorders, including *C9orf72*-ALS (*C9orf72*-associated amyotrophic lateral sclerosis) or *C9orf72*-FTD (*C9orf72*-associated frontotemporal dementia). OH received grant support from the Science Foundation Ireland and Health Research Board; consulting fees from Biogen and Wave Pharmaceuticals; performs editorial duties for Taylor and Francis; and served on the data safety board for MediNova and advisory board for Novartis. AMI received funding from the UK Dementia Research Institute, principally funded by the MRC, and additional funding partners LifeArc and ARUK; and is a co-inventor on UK (2105455.6) and international patents (PCT/EP2022/060296) for "CasRx/Cas13d systems targeting C9orf72" to target both sense and antisense C9orf72 repeats. MvB receives funding from NINDS (RF1 NS123052 and R01 NS121125) and the Spastic Paraplegia Foundation. RR received funding from NIA and the NINDS (U19 AG063911 and UG3 NS103870), the US Department of Defense, The Fund Generet, and the Fund for Scientific Research Flanders; and is an author on a patent entitled: "Detecting Frontotemporal Dementia and Amyotrophic Lateral sclerosis" (US 14343807). All other authors declare no competing interests.

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