C9orf72 expansions in frontotemporal dementia and amyotrophic lateral sclerosis

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***C9orf72* hexanucleotide repeat expansions are the most common cause of familial frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) worldwide. The clinical presentation is often indistinguishable from classic FTD or ALS, although neuropsychiatric symptoms are more prevalent and, for ALS, behavioural and cognitive symptoms occur more frequently. Pathogenic repeat length is in the hundreds or thousands, but the minimum length that increases risk of disease, and how or whether the repeat size affects phenotype, are unclear. Like in many patients with FTD and ALS, neuronal inclusions that contain TARDBP are seen, but are not universal, and the characteristic pathological finding is of dipeptide repeat (DPR) proteins, formed by unconventional repeat-associated non-ATG translation. Possible mechanisms of neurodegeneration include loss of C9orf72 protein and function, RNA toxicity, and toxicity from the DPR proteins, but which of these is the major pathogenic mechanism is not yet certain.**

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Introduction

The discovery in 2011 that hexanucleotide repeat expansions in the *C9orf72* gene were a common cause of both frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) was a crucial point in neurodegenerative disease research.[1,2] These two disorders have been known to occur within the same family, and even within the same person, but *C9orf72* expansions provided an incontrovertible molecular link. Although frequency varies geographically, expansions in *C9orf72* account for a high proportion of patients with familial FTD and ALS in most countries, and are also described in a substantial number of apparently sporadic cases.[3,4]

In the past 3 years, research into FTD and ALS has accelerated, with increasing collaboration across the specialties and major advances in our knowledge of both disorders. However, many questions about *C9orf72*-related neurodegeneration remain unanswered. Although most patients have a *C9orf72* expansion[1,2,5,6] that is unambiguously in the pathogenic size range, the minimum repeat length that confers increased risk is uncertain, as is any relation between repeat length and clinical phenotype. Although most patients present clinically with behavioural variant FTD (bvFTD) or ALS,[1,2] the phenotypic range encompasses rapidly and very slowly progressive disease and clinical presentations that differ significantly from classic FTD or ALS.[6] Pathologically, neuronal inclusions containing TARDBP (also known as TDP-43) are seen, similar to other causes of FTD and ALS, but the unique characteristic of *C9orf72* expansions is the presence of inclusions containing dipeptide repeat (DPR) proteins.[7,8] Furthermore, the mechanism by which *C9orf72* expansions cause neurodegeneration is controversial, and both loss-of-function and gain-of-function mechanisms have been proposed.[9]

In this Review, we pose some key questions in *C9orf72*-related neurodegeneration research, and outline knowledge and future directions that might help to resolve them.

What is the minimum repeat length that confers risk of disease?

The *C9orf72* gene on chromosome 9 has three transcription variants, and two possible protein isoforms. A hexanucleotide GGGGCC (G4C2) repeat region is located either in the promoter or in intron 1 of the gene, depending on the transcript variant (figure 1). The normal repeat size is variable, with more than 90% of the European population having between two and ten G4C2 repeat units.[1] Larger repeats are less common, and repeats of more than 30 units in length are an important, albeit rare, finding in healthy populations.[1,2,5,6,10] Repeat expansions typically seen in patients are far larger than this normal range, consisting of at least several hundred or, more often, thousands of repeats.[2,6,11] No strong evidence exists for intergenerational anticipation.

Although an expansion of several hundred units or more is clearly pathogenic, we still do not know the smallest hexanucleotide expansion that confers a risk. For example, in a patient with familial ALS, an expansion of around 90 units in blood was associated with a massive expansion in the brain, which was highly likely to be causal.[12] Expansions of between 20 and several hundred units have been reported both in patients with FTD or ALS[13–17] and in healthy controls,[6,18] and might be termed intermediate because the evidence for pathogenicity is unclear. The instability of the repeat in somatic tissues, which results in a range of mutation sizes in particular tissues and variation between tissues, means that intermediate repeats detected in blood-sample-derived DNA might be associated with massive expansions in the brain. Alternatively, intermediate repeats might be associated with the same loss-of-function or gain-of-function mechanisms that have been proposed for the massive expansions.[5] This issue is important for genetic counselling, and is also important when considering the requirements of technology for accurate genetic diagnosis. The widely used screening technology, which uses repeat-primed PCR, cannot reliably distinguish between repeat sizes larger than around 30–50 units, and is therefore not

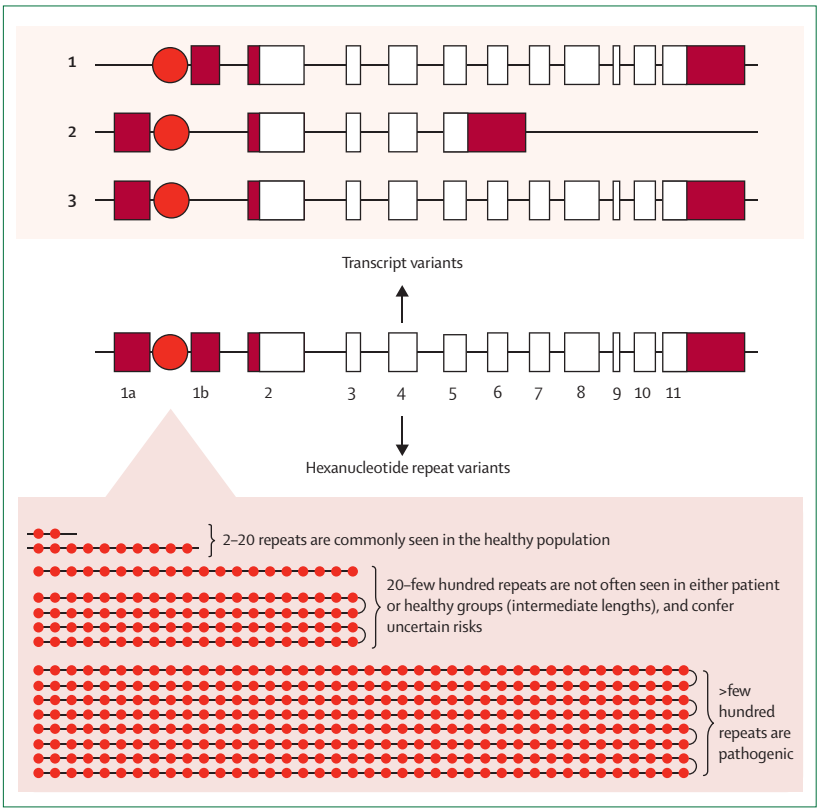

Figure 1: **The *C9orf72* gene and transcripts**
A schematic diagram of the *C9orf72* gene, with three transcript variants shown above (variants 2 and 3 may lead to hexanucleotide repeat transcription), and hexanucleotide repeat lengths shown below. Exons are shown boxed, and untranslated regions are coloured. The repeat region is denoted by a red circle. Typical repeat lengths in the clearly healthy range, an intermediate range of uncertain risk, and large pathogenic expansions are shown as chains of red circles. Repeat lengths of between 20 and 30 are, in most studies, shown to be uncommon alleles in European-derived populations that confer no additional risk of frontotemporal dementia or amyotrophic lateral sclerosis.

adequate when used without confirmation of a large expansion by Southern blot.[1,2] Furthermore, results of a masked study at 14 laboratories showed a lack of accuracy when repeat-primed PCR was used in isolation, and the authors recommended that use of Southern blot and PCR should be obligatory in a clinical diagnostic setting.[19]

Do carriers of the *C9orf72* expansion arise from a single common founder?

The strong association of the expansion mutation with a 20-single nucleotide polymorphism (SNP) haplotype, and the high prevalence of the mutation in Finland, understandably led to speculation about a single founder of worldwide cases, disseminated by the Vikings.[20,21] However, the high prevalence in some southern European populations, and occurrence in distant and large populations in East Asia is difficult to explain with Viking emigration.[22,23] The 20-SNP haplotype is common, associated with more unstable, longer normal-range repeats, and is likely to have an ancient, out-of-Africa origin because it is found in European, African, and Asian populations.[6,24] The finding of high mutation frequencies in particular regions or groups that have undergone marked contractions and re-expansion in population size has many precedents, and does not in itself implicate a single founder of all cases worldwide.[25–27] The alternative hypothesis of a predisposing haplotype that has generated many mutations is viable, and prompts further investigation of cis-acting factors other than increasing repeat length, which might make the repeat region more unstable.[5] The description of expansions increasing from about 100 to more than 1000 repeats, both in one generation[15] and somatically in the CNS,[11,12] supports the hypothesis that many events occur on a predisposing haplotype.

What clinical phenotypes are associated with *C9orf72* expansions?

The most common clinical phenotypes associated with *C9orf72* expansions are bvFTD,[28–35] ALS,[35–41] or the combination of both in one person. However, other phenotypes have been described, and even cases described as classic bvFTD or ALS have extensive heterogeneity (panel 1).

The most common clinical syndrome within the FTD clinical range is bvFTD, and diagnostic criteria include five clusters of behavioural symptoms: apathy; disinhibition, and socially inappropriate behaviour; abnormal eating behaviour; loss of empathy; and perseverative, stereotyped, or obsessive-compulsive behaviour.[42] However, a few patients with bvFTD have been known to develop other changes in behaviour, including features of psychosis (hallucinations, delusions, or both). Patients who develop hallucinations and delusions are clearly over-represented in *C9orf72*-associated bvFTD compared with those without *C9orf72* expansions: in large series, 28–56% of patients with *C9orf72* expansions had hallucinations and delusions compared with 4–18% of patients without the expansion.[29–35,43–45] These symptoms can even predominate at onset and lead to a diagnosis of obsessive-compulsive disorder,[46] schizophrenia,[47] bipolar disorder,[48,49] or depressive pseudodementia,[50] at least in the early stages of disease.

The sixth major diagnostic criterion for bvFTD is the presence of executive dysfunction as the main form of cognitive impairment.[42] However, although executive function is often the predominant cognitive domain involved in bvFTD, this is not universal, and other domains might be involved in some patients, including episodic memory at onset. In these cases, the differential diagnosis from typical Alzheimer's disease becomes difficult, and this amnestic phenotype has been described in association with *C9orf72* expansions.[51–55] However, this issue is not unique to *C9orf72* expansions, and has likewise been described for both *MAPT* and *GRN* mutations[52] (table). Impaired episodic memory in the *C9orf72* expansion group might correlate with brain atrophy of the posterior cingulate gyrus and parietal lobes, in addition to frontal and temporal areas identified in other types of FTD.[56]

Overall, these neuropsychiatric and amnestic features mean that patients with *C9orf72* expansions who present with a progressive behavioural-cognitive phenotype are less likely to meet diagnostic criteria for bvFTD than those without the expansion,[34] complicating both the clinical diagnosis and the formulation of consensus research guidelines. Some symptomatic mutation carriers might be diagnosed with Alzheimer's disease, and might not get referred for genetic testing.[6,51–53]

The phenotypic range of FTD also includes language-led syndromes—the primary progressive aphasias (PPAs)—which comprise three major subtypes: non-fluent variant (nfvPPA; also known as progressive non-fluent aphasia), semantic variant (svPPA; also known as semantic dementia), and logopenic variant (lvPPA; also known as logopenic aphasia).[57] PPA is a fairly rare phenotype of *C9orf72* expansions and, although two early papers described a substantial proportion of cases (27% with nfvPPA in a Finnish cohort;[1] and 14% with unclassified PPA, and 5% with svPPA in a Dutch cohort[18]), other large series have described only a few cases (all with an svPPA or nfvPPA phenotype),[29,30,58] and some described no cases at all.[31,32,34] One case report described an association of lvPPA with a *C9orf72* expansion; however, this patient also had evidence of underlying amyloid pathology.[59]

When patients with *C9orf72* expansions present with ALS as the initial clinical syndrome, it is often indistinguishable from classic ALS. Results of initial studies suggested that bulbar symptoms were more common than limb symptoms at onset,[36,38,39] but results of subsequent cohort studies have not corroborated this.[37,40] Cognitive or behavioural impairment, or both, seem to be much more common in patients with ALS with *C9orf72* expansions than in those without expansions (either sporadic ALS or with mutations in other genes), occurring in nearly half of cases.[41,60] *C9orf72* expansions seem to only rarely cause progressive muscular atrophy or primary lateral sclerosis.[61]

The association of FTD and ALS in the same patient is not restricted to those with *C9orf72* expansions. Results of a neuropsychological study comparing patients with and without *C9orf72* expansions were similar in both groups, but neuropsychiatric features were more common in the *C9orf72* group, with a trend towards more frequent bulbar features.[62]

Several groups have investigated whether *C9orf72* expansions are present in other neurodegenerative disorders. Parkinsonism can be a feature of FTD syndromes, and has previously been reported in association with *MAPT* and *GRN* mutations[63] (table). Similarly, parkinsonism is sometimes seen in patients with *C9orf72* expansions who initially present with FTD. However, various parkinsonian phenotypes in which *C9orf72* expansions have been rarely described in the absence of FTD or ALS include Parkinson's disease,[64–68] dementia with Lewy bodies,[69] multiple system atrophy,[70] and corticobasal syndrome.[71] *C9orf72* expansions have also

***Panel 1*: Representative case histories of patients with *C9orf72* expansions**

Case 1

A 70-year-old man presented with a 2-year history of progressive change in personality. He had become more apathetic and was often disinhibited, saying inappropriate things. He had developed paranoid delusions about people living in his street, and had become repetitive in his behaviour, sticking to a rigid routine throughout the day. His mother was said to have become odd at around the age of 57 years, and suddenly became verbally aggressive with people. A few years later she started to have difficulty walking and was diagnosed with amyotrophic lateral sclerosis (ALS), eventually dying at the age of 66 years. His maternal aunt died of ALS in her 80s, and his elder sister had a diagnosis of frontotemporal dementia (FTD)-ALS, with symptoms starting in her 60s. Examination showed that he had widespread fasciculations in the upper and lower limbs without any other abnormalities. Neuropsychometric testing revealed executive dysfunction, but other cognitive domains were intact. He was seen 6 months later, by which time he had become dysphagic and dysarthric; examination revealed mixed bulbar and pseudobulbar features, widespread wasting and fasciculations in the limbs, and brisk deep tendon reflexes. He continued to deteriorate, and died 18 months later.

Case 2

A 36-year-old woman presented with progressive weakness in both legs during the previous 2 years, and with dysarthria and dysphagia during the past year. She had a normal cognitive examination, but had a bulbar dysarthria, a wasted tongue with fasciculations, and a brisk jaw jerk. She had evidence of wasting and fasciculations in the limbs, particularly in the quadriceps. All four limbs had spasticity, with evidence of symmetrical pyramidal weakness. Her symptoms deteriorated during the next year and, following a few admissions with aspiration pneumonia, a percutaneous endoscopic gastrostomy was placed for feeding. Her father (who was in his 60s) was diagnosed at this time with motor neuron disease. She continued to deteriorate, and was admitted to a hospice 2 years later.

Case 3

A 60-year-old man presented with a 4-year history of progressive memory impairment. He was unable to recall peoples' names or things he had done recently. He also started to get lost in familiar environments. His wife said that, during this time, he had become more withdrawn and apathetic. His mother had been under a Mental Health Act section for paranoid behaviour in her 70s, and his maternal aunt had been diagnosed with late-onset schizophrenia. His half-sister (with the same mother) had learning disabilities from an early age. His mini mental state examination (MMSE) score was 27/30, with a normal neurological examination. Formal neuropsychological assessment showed clinically significant executive dysfunction and mildly impaired episodic memory, with other cognitive domains preserved. CSF MAPT was 217 pg/mL (normal range up to 300 pg/mL), and Aβ42 was 556 pg/mL (normal range >222 pg/mL).

Case 4

A 59-year-old man presented with an 11-year history of slowly progressive behavioural change. His wife said that he had become slowly more difficult to live with and less motivated to do things around the house or to go out socially. He had become fixed in the way that he did things. His MMSE score was 30/30, and more detailed cognitive and neurological examinations were normal. Neuropsychometric testing was also entirely normal. He was followed up yearly throughout the next 10 years. His wife reported that his behaviour was becoming slowly worse, but testing showed no observable abnormalities: he scored 30/30 on the MMSE at each visit, and neuropsychometric testing was normal. MRI scans of his brain were reported as normal. His behavioural symptoms were dominated by apathy and mental rigidity, but in the past couple of years he had become more obsessional, and developed a sweet tooth with associated weight gain. Reviewing his family history, his mother had started to behave oddly at around the age of 63 years and, although this deteriorated with time, progression was slow, and she lived until the age of 87 years; his maternal uncle, however, had ALS at the age of 31 years, and died at the age of 34 years.

	C9orf72	*MAPT*	*GRN*
Clinical features			
Main syndrome	bvFTD/ALS/FTD-ALS >PPA	bvFTD>CBS>PSP syndrome	bvFTD>PPA>CBS
ALS	Common	Not reported	Rare
Parkinsonism	Can occur, but usually fairly late; some patients described with primary parkinsonian disorder	Can occur and might be early in the illness; some patients have a corticobasal syndrome or, less often, a PSP phenotype	Can occur, but usually fairly late; some patients have a corticobasal syndrome
Cerebellar features	Reported in a few cases	Not reported	Not reported
Neuropsychiatric and behavioural features			
Behavioural abnormalities	Apathy, disinhibition, loss of empathy	Disinhibition, abnormal eating behaviour	Apathy, disinhibition, abnormal eating behaviour
Hallucinations and delusions	Hallucinations and delusions fairly common	Uncommon	Hallucinations seen in some patients
Cognitive features			
Executive dysfunction	Common	Common	Common
Language impairment	Small number of patients reported with a progressive aphasia, mostly non-fluent	Patients can develop semantic impairment but usually following behavioural symptoms, and non-fluent aphasia very rare	Some patients have a progressive aphasia—prominent anomia, non-fluent speech
Memory impairment	Can occur early in the disease (often leading to a clinical diagnosis of Alzheimer's disease)	Often later in the illness, but can occur early	Usually late in the illness, but can be prominent in some cases
Parietal lobe dysfunction	Seen in some patients, particularly as the disease progresses	Can occur late in the disease	Seen fairly commonly, particularly as the disease progresses
Imaging features			
Symmetry	Often fairly symmetrical atrophy	Often fairly symmetrical atrophy	Asymmetrical atrophy, either right or left predominant
Areas involved	Variable: fronto-insular atrophy with temporal and parietal involvement; thalamus and cerebellar atrophy also seen	Anterior temporal and orbitofrontal atrophy	Temporo-fronto-parietal atrophy

FTD=frontotemporal dementia. bvFTD=behavioural variant FTD. ALS=amyotrophic lateral sclerosis. PPA=primary progressive aphasia. CBS=corticobasal syndrome. PSP=progressive supranuclear palsy.

Table: **Comparison of features of FTD associated with *C9orf72* expansions, *MAPT* mutations, and *GRN* mutations**

been reported in hyperkinetic disorders, with one study identifying them in 2% of patients who tested negative for Huntington's disease expansions (ten of 514 cases).[72] Although not studied in detail, a few cases of cerebellar ataxia associated with *C9orf72* expansions have been reported in the scientific literature.[71]

What is the prognosis of *C9orf72*-associated disease?

ALS and FTD-ALS are usually rapidly progressive neurodegenerative disorders, and survival from symptom onset is often a few years. Although many cases of *C9orf72*-associated FTD or ALS are similarly rapidly progressive, there are increasing numbers of reports of patients with slow progression and prolonged survival for 20 or more years.[73–75] Many of these cases present clinically with bvFTD, and have been previously thought to fit into the so-called bvFTD phenocopy group, for whom the underlying pathogenesis has so far been unclear. *C9orf72* expansions might account for a proportion of bvFTD phenocopies; these patients might have a previously undisclosed family history of ALS, providing a clue for *C9orf72* screening.

What modifies the phenotype and progression of *C9orf72*-related neurodegeneration in such a profound way? Although results of one study suggested that longer repeats in the cerebellum were associated with poorer survival,[11] the relation between expansion length and phenotype is unclear. Genetic modifiers of *C9orf72* expansions have been proposed, including *TMEM106B*,[76,77] which has been associated with a later age at onset and death in patients with FTD, and *ATXN2*,[78] which might predispose patients to development of ALS rather than FTD.

How should clinicians counsel people at risk for a *C9orf72* expansion?

Two key results from studies of *C9orf72* are particularly pertinent to clinical genetic practice:[79,80] first, the presence of expansions in a substantial proportion of patients with sporadic FTD and ALS;[1–3] and second, the presence of two pathogenic mutations in one patient.[81–86] All patients with FTD or ALS who have a clear autosomal dominant family history should certainly undergo genetic screening, but should clinicians now counsel all patients with sporadic FTD or ALS, and test for *C9orf72* with consent? Clinical features such as the combination of FTD and ALS, delusions or hallucinations, and hints of a family history, might enrich cases, but even unselected sporadic cases are significantly prevalent (up to around 10% of FTD and ALS cases). However, this creates several challenges for counselling: we do not have a satisfactory understanding of the concurrence of pathogenic mutations, or accurate data on penetrance or the usefulness of phenotypic predictors.[81–86] These uncertainties might, in time, be resolved by prospective genetic studies of gene carriers, but this should not deter discussion of genetic risk with patients presenting with apparently sporadic disease.

Is there a specific neuroimaging phenotype of *C9orf72* expansions?

Unlike FTD associated with *MAPT* or *GRN* mutations, *C9orf72* expansions do not have a characteristic neuroimaging signature in the individual patient: structural MRI reveals variable patterns of cortical and subcortical atrophy[29–35] (table). In line with this finding, results of cohort studies using voxel-based morphometry have shown an extensive atrophy profile involving distributed frontal, insular, temporal, and parietal cortical and subcortical regions, including the thalamus and cerebellum, compared with healthy age-matched controls and other FTD subtypes[29,87,88] (figure 2A). Atrophy in patients with *C9orf72* expansions is more symmetrical than in those with *GRN* mutations, and there is less temporal lobe involvement

than in patients with *MAPT* mutations.[29,87] Patients presenting with *C9orf72*-associated ALS show more extensive involvement of non-motor frontal cortical areas and basal ganglia structures than patients with ALS who do not have a *C9orf72* expansion.[38,89] Compared with other genetic forms of FTD, subcortical (in particular, thalamic and cerebellar) involvement is relatively prominent across studies, in accordance with neuropathological evidence, and might constitute a group-level signature of *C9orf72* expansions. This conclusion is supported by data for longitudinal atrophy profiles showing preferential volume loss most consistently in the thalamus and cerebellum in symptomatic *C9orf72* expansion carriers with FTD.[90] However, some patients (in particular, those previously thought to be bvFTD phenocopies) show little progression of atrophy even after prolonged intervals[73] (figure 2B).

A recent study investigating structural imaging in asymptomatic family members carrying *C9orf72* expansions has suggested that atrophy can be seen up to 25 years before predicted symptom onset. Clinically significant volume differences were seen initially in subcortical areas including the thalamus, as well as the insula and more posterior cortical areas, followed by later involvement of the frontal and temporal lobes and cerebellum.[91] The reason for this very early presymptomatic change is unclear, and although it could be consistent with the slowly progressive atrophy seen in some cases, another possibility is that the observed change is due to longstanding developmental abnormalities with superimposed atrophy being seen only later on.

Data for white matter pathways in patients with *C9orf72* expansions are scarce, but are broadly concordant with grey matter atrophy profiles: diffusion tractography has implicated long intrahemispheric, commissural, and corticospinal tracts in FTD,[29] whereas patients presenting with ALS show more involvement of frontal and temporal lobe white matter tracts than those without the mutation.[92]

At present, little information is available about functional neuroimaging correlates of *C9orf72* expansions: results of fluorodeoxyglucose (FDG)-PET and SPECT studies have shown frontal, temporal, and parietal cortical and subcortical alterations that are broadly concordant with the distribution of structural damage,[35,93] and that are more marked in patients with ALS associated with *C9orf72* expansions than in patients without the mutation.[94]

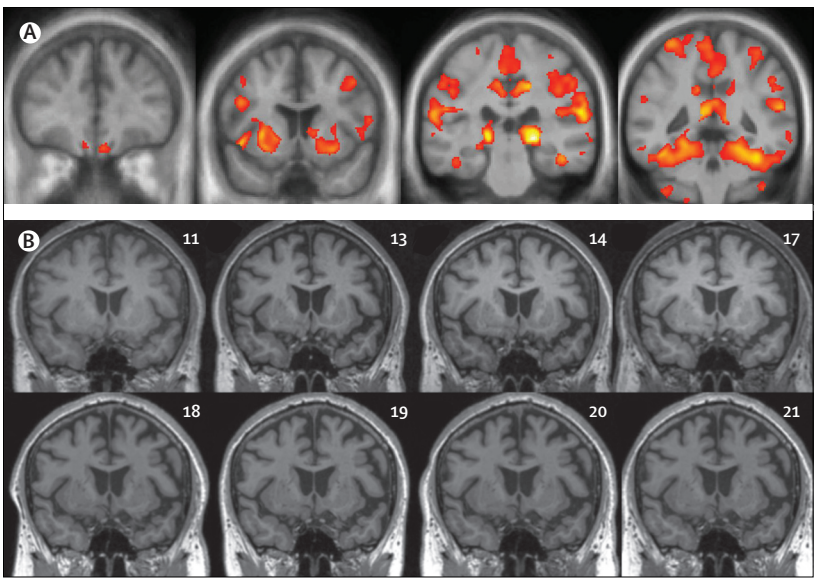

Figure 2: **Neuroimaging of *C9orf72* expansions**
(A) Voxel-based morphometry analysis of MRI scans from 14 patients with *C9orf72* expansions and behavioural variant frontotemporal dementia (bvFTD) (mean disease duration 4·9 years [SD 3·2], mean age at scan 59·9 years [SD 8·2]) compared with 50 age-matched cognitively normal control individuals (mean age at scan 60·2 years [SD 13·8]), with a false discovery rate corrected for multiple comparisons <0·05 and adjusted for age and sex, shows the most substantial involvement in the frontal-insular regions, thalamus, cerebellum, and also in the parietal lobes. (B) Eight serial coronal T1 MR brain scans from a patient with FTD and a *C9orf72* expansion who had slowly progressive disease (described in panel 1, case 4). The initial scan was taken 11 years after symptom onset (numbers represent years from symptom onset). His scans were reported as normal on visual inspection. Volumetric analysis revealed a whole brain volume at the initial scan of 1333·8 mL and at the final scan of 1192·1 mL, giving an annualised rate of brain atrophy of 1·1% per year throughout the 10 years, compared with a mean rate of 0·6% (SD 0·9) per year in 12 age-matched controls (mean age at first scan 59·0 years [SD 4·9]).

What are the physiological mechanisms underlying the clinical presentation of *C9orf72* expansions?

The neuromuscular physiology of *C9orf72* expansions has not been studied systematically or in detail. Available reports describe widespread denervation and intact sensory responses consistent with a diagnosis of ALS.[64,70,95,96] Clinical experience, however, suggests that at least some patients presenting with FTD will have fasciculations despite normal electrophysiological results.

The systems pathophysiology of *C9orf72* mutations has been studied with a novel somatosensory framework based on body schema integration.[97,98] Body schema processing is likely to be a key function of the distributed cortico-thalamo-cerebellar network implicated in group studies of *C9orf72* expansions,[29,87,88,90] and derangements of body schema integrity are relevant to the prominent neuropsychiatric symptoms these patients frequently describe.[97] Patients with FTD associated with *C9orf72* expansions show abnormalities of various aspects of body schema processing, including tactile discrimination, body-part illusions, and self/non-self differentiation, compared with both healthy older individuals (aged >60 years) and patients with other forms of FTD, suggesting that this might be a candidate pathophysiological substrate for the clinical phenotype in these cases.[98]

What are the key pathological features of *C9orf72* expansions?

In line with the clinical presentation, the pathological phenotype of patients with *C9orf72* expansions is usually frontotemporal lobar degeneration (FTLD), ALS, or a combination of both. FTLD is a term used to describe the pathological findings frequently seen in patients with the clinical diagnosis of FTD, and includes three major subtypes defined by inclusions containing the proteins MAPT, TARDBP, or FUS.[99,100] Most *C9orf72* cases are

characterised by underlying TARDBP pathology, irrespective of their clinical phenotype.[28–39,101–103] In FTLD, a relatively symmetrical pattern of atrophy is seen in patients with *C9orf72* expansions compared with other forms of FTLD-TARDBP,[84] with the frontal and temporal cortices, hippocampus, pyramidal motor system, amygdala, striatum, thalamus, and midbrain (including the substantia nigra) affected by TARDBP pathology.[28,101] Patients with a clinical phenotype of FTD in the absence of ALS show significantly less degeneration and TARDBP pathology in lower motor neurons compared with those with a mixed FTD-ALS clinical phenotype. Four FTLD-TARDBP subtypes have been described (A–D) on the basis of the underlying morphological phenotypes of the pathological inclusions, and patients with *C9orf72* expansions have been shown to have pathological lesions in keeping with subtypes A and B.[29,85] FTLD-TARDBP type A lesions consist of compact or granular neuronal cytoplasmic inclusions (NCIs), dystrophic neurites, and occasional neuronal intranuclear inclusions (figure 3A, figure 3B). The pathology is usually found in layer 2 of the cortex, and can also be found in the cortical white matter (figure 3C). Patients presenting with FTLD-TARDBP type B pathology have granular NCIs in all cortical layers, and few dystrophic neurites and neuronal intranuclear inclusions (figure 3D). Single cases of *C9orf72* expansions have been reported with an underlying FTLD-TARDBP type C pathology characterised by long corkscrew neurites and occasional NCIs,[101,104] and even cases that lack TARDBP pathology have been reported.[30] Cases of *C9orf72* expansion with an ALS clinical phenotype are pathologically indistinguishable from typical sporadic ALS, with predominant degeneration and TARDBP-positive NCIs of variable morphology in upper, lower, brainstem, and spinal cord motor neurons. The extramotor cortices, hippocampi, and subcortical regions are usually mildly affected.[39] Cases of a mixed FTD-ALS clinical phenotype have a combination of TARDBP pathology with more severe degeneration and greater amounts of TARDBP pathology in the frontal and temporal cortex (fitting into the FTLD-TARDBP type B subgroup).

In addition to TARDBP pathology, a unique and highly characteristic pathological feature of *C9orf72* cases is the presence of SQSTM1-positive NCIs in the hippocampal granule cell layer, cerebellum, and neocortical neurons (figure 3E–G). These NCIs are negative for TARDBP, and have a unique star-like morphology.[29,85,105,106] Results of studies have shown that these inclusions are composed of unconventional DPR proteins formed from translation of the abnormally expanded repeat in *C9orf72*.[7,8,107–110] Novel antibodies raised against the dipeptides label the SQSTM1-positive star-like inclusions. This highly specific pathology associated with the *C9orf72* expansion repeat, in some studies, helped to identify families carrying two known pathogenic mutations,[85] and has led to the suggestion that *C9orf72* expansion cases could be reclassified as FTLD-DPR, since cases can fit into one of two FTLD-TARDBP subtypes, or might even lack TARDBP pathology.[8,30]

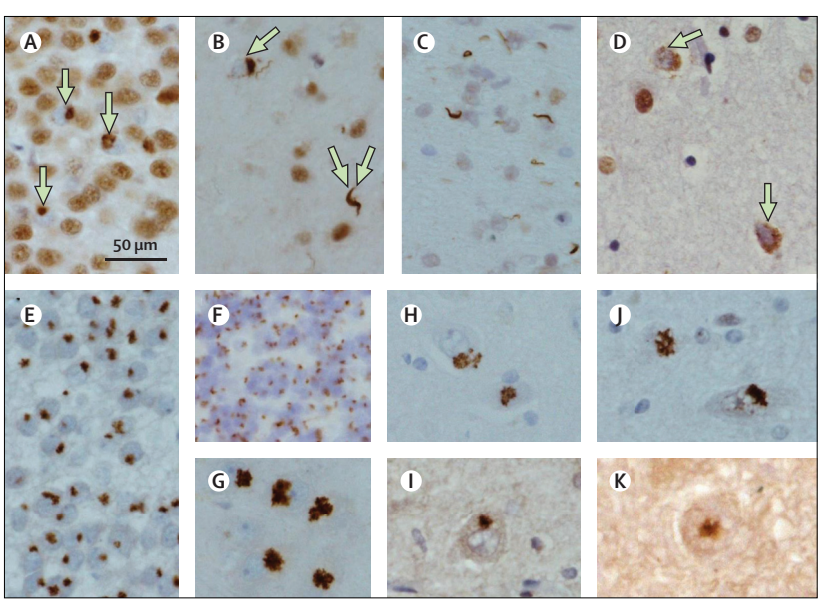

Figure 3: **Pathology of *C9orf72* expansions carriers**
(A) TARDBP immunohistochemistry highlights compact neuronal cytoplasmic inclusions (arrows) in the granule cell layer of the hippocampus in all frontotemporal lobar degeneration (FTLD)-TARDBP subtypes. (B) Compact TARDBP neuronal cytoplasmic inclusions (arrow), and short dystrophic neurites (double arrow) as seen in FTLD-TARDBP type A. (C) TARDBP pathology is also evident as neuropil threads and glial inclusions in the white matter of FTLD-TARDBP type A. (D) Granular neuronal cytoplasmic inclusions are observed in the deeper cortical layers of FTLD-TARDBP subtype B (arrows). SQSTM1-positive star-like neuronal cytoplasmic inclusions indicative of a *C9orf72* expansion repeat, which are negative for TARDBP pathology, are seen in the hippocampal granule cell layer (E), cerebellum (F), and CA4 subregion of the hippocampus (G). The SQSTM1-positive inclusions are also composed of unconventional dipeptides formed from the translation of sense and antisense transcripts of the abnormally expanded repeat in *C9orf72*. Translation of the expanded transcripts generates five different polypeptides, each composed of repeating units of two aminoacids: glycine-proline (H), glycine-alanine (I), glycine-arginine (J), alanine-proline (K), and arginine-proline (not shown).

By what mechanisms do *C9orf72* expansions cause neurodegeneration?

The mechanism of neurodegeneration in *C9orf72* expansions is unclear, but is likely to occur through loss of C9orf72 protein, gain-of-function mechanisms, or both (figure 4). All three transcript variants of *C9orf72* have been shown to be decreased in blood,[109,110] cortex,[2,103,111,112] cerebellum,[111,112] spinal cord,[112] and patient-derived stem cells differentiated into neurons[112] from patients carrying *C9orf72* expansions compared with healthy controls or patients without a repeat expansion in *C9orf72*. This finding is at least partly attributable to hypermethylation of a CpG island upstream of the repeat,[113–115] and trimethylation of lysine residues on H3 and H4 histones, which bind G4C2 repeats and suppress transcription.[110] In patient-derived cells, demethylation leads to increased vulnerability to external stressors, suggesting that reduction of mutant *C9orf72* transcript numbers by methylation could be an innate protective mechanism.[115]

Results of studies have shown that C9orf72 is highly homologous to differentially expressed in normal and neoplasia (DENN) proteins, which function as guanine

nucleotide exchange factors (GEFs) to activate RAB GTPases and regulate membrane trafficking.[116,117] Consistent with this, knockdown of C9orf72 protein in neuronal cell lines leads to defects in endocytosis and autophagy.[118] Knockdown or knockout of *C9orf72* in zebrafish and worm models show axonopathy and motor dysfunction,[119,120] but this has not been replicated in mice.[121] The role of *C9orf72* loss of function is therefore unclear.

Novel species that result from the expanded repeat include repeat RNA[122–124] and, as discussed above, DPR proteins generated by repeat-associated non-ATG (RAN) translation. Repeat RNA forms frequent sense and antisense neuronal RNA foci in brain regions affected by disease,[107,121,125,126] and the burden in these brain regions correlates with clinical phenotypes.[125] The classic hypothesis for non-coding repeat expansion disorders is that RNA foci sequester essential RNA-binding proteins. Sense G4C2 repeats have been shown to bind to multiple proteins, including many splicing factors and, although variable, sequestration of some of these proteins into foci has been observed.[112,127–133] However, further work is needed to elucidate the role of RNA-binding protein sequestration in disease pathogenesis.

RAN translation of the *C9orf72* repeat can occur in all six sense and antisense frames, resulting in five different DPR proteins, composed of glycine-alanine (GA), glycine-proline (GP), and glycine-arginine (GR) in the sense frames, and glycine-proline (GP), alanine-proline (AP), and proline-arginine (PR) in the antisense frames. As discussed above, all of these DPRs form neuronal inclusions in the brain (figure 3H–K) that colocalise with SQSTM1-positive (but not TARDBP-positive) pathology. A detailed analysis of the neuroanatomical distribution of pathology shows that TARDBP inclusions correlate with neurodegeneration, but poly-(GA) inclusions do not.[134]

Overexpression of expanded G4C2 repeats (not within the context of the *C9orf72* gene) has been shown to exert toxicity in cell lines,[126] flies,[133,135] and zebrafish,[130] suggesting that gain-of-function mechanisms are sufficient for neurodegeneration. Results of studies show that specific DPR proteins have an important role in this process. In cultured neurons, expression of a poly-GA peptide results in cytoplasmic aggregate formation, reduced neuritic branching, and apoptosis.[136,137] Molecular methods that enabled study of G4C2 repeat RNA and DPR proteins revealed that the arginine-rich DPR proteins poly-(GR) and poly-(PR) are mainly responsible for G4C2 repeat-induced neurodegeneration in *Drosophila*.[134] Furthermore, the arginine-rich DPR peptides GR and PR are retained in the nucleoli of cells, and cause transcriptional dysregulation and toxicity.[138] The results of these new studies show the importance of specific DPR proteins in *C9orf72*-associated FTD-ALS pathogenesis.

Antisense oligonucleotides targeting the sense strand of *C9orf72* have been shown to reduce RNA foci and protect against excitotoxicity,[112,121,132] suggesting that antisense oligonucleotides are a potential therapeutic strategy for *C9orf72*-associated FTD and ALS. Small molecules that target secondary structures formed by *C9orf72* repeat expansion RNA have also been shown to inhibit RNA foci formation and RAN translation in a cell line, and in neurons derived from patients with *C9orf72*-associated ALS.[139]

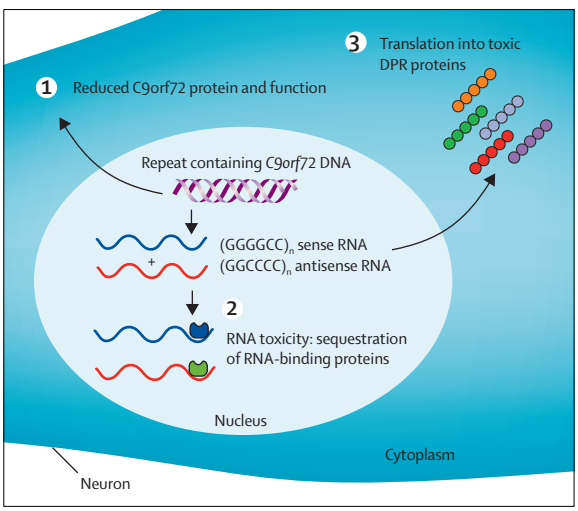

Figure 4: **Potential mechanisms of neurodegeneration in *C9orf72* expansions**
Potential mechanisms of neurodegeneration in *C9orf72* expansions include: loss of function (1), RNA toxicity (2), and toxicity from dipeptide repeat (DPR) proteins (3).

In summary, evidence suggests that both loss-of-function and gain-of-function mechanisms result from the *C9orf72* expansion, but the relative contribution of these mechanisms and the molecular pathways that lead to neurodegeneration are yet to be identified. The specific roles of sense and antisense repeat RNA and DPR proteins are likewise yet to be elucidated.

Is there a viable cell model of *C9orf72* expansions?

The development of robust cell models that faithfully recapitulate key features of the disease in the relevant cell types will be crucial to elucidate the molecular mechanisms underlying neuronal death in this disease. The wide range of pathogenic G4C2 repeat sizes and the large size of the repeat region, combined with the fact that it is entirely GC, mean that it is not amenable to cloning via conventional PCR-based strategies, making the generation of cell models technically difficult. Differentiation of patient-derived induced pluripotent stem cells (iPSCs) into motor neurons, cortical neurons, or both is therefore a promising approach for disease modelling. This technique can recapitulate key disease features, including RNA foci in *C9orf72* neurons.[112,126,131] The presence of RNA foci led to the sequestration of RNA-binding proteins ADARB2,[107] HNRNPA1, and PURA,[132] supporting the hypothesis that disrupted RNA metabolism is central to disease pathogenesis. Indeed, results of transcriptomics of *C9orf72* iPSC neuronal cultures show a dysregulated gene expression signature similar to *C9orf72*

Panel 2: Priorities for research in *C9orf72*-related FTD and ALS

- Clarification of the minimum length of repeat expansion in blood (and brain) that confers increased risk
- Development of, and research into the effect of, guidelines for genetic counselling of patients with sporadic FTD or ALS
- Further discovery of genetic modifiers that lead to the variable clinical phenotype
- Investigation of the cortico-thalamo-cerebellar network that has been implicated in neuroimaging and pathophysiology studies
- Understanding the major mechanisms of neurodegeneration, including the specific roles of sense and antisense repeat RNA and dipeptide repeat proteins
- Investigation of the role that TARDBP has in *C9orf72* expansions, since most, but not all, cases have TARDBP pathology
- Development of robust cell models of *C9orf72* expansions
- Discovery of biomarkers of disease onset through investigation of pre-symptomatic cohorts of at-risk family members
- Discovery of biomarkers of disease progression that can be used as outcome markers in clinical trials
- Screening of drug candidates that can be used in pilot clinical trials

FTD=frontotemporal dementia. ALS=amyotrophic lateral sclerosis.

Search strategy and selection criteria

We searched PubMed (from Jan 1, 1966, to June 30, 2014) with the search term "*C9orf72*" in all languages. Further articles were included from reference lists, review articles, and major textbook chapters. Abstracts from relevant meetings were also included. The final reference list was generated on the basis of originality and relevance to the topics covered in this report.

For the **Genetic FTD Initiative** see http://www.genfi.org.uk

tissue.[112,132] One study focused on CNS genes that have a secreted protein product, and identified seven genes that were dysregulated in both iPSC neurons and tissue, with a view to development of these as potential CSF biomarkers.[112] Results of another study showed dysregulation of several genes linked to membrane excitability, and this was accompanied by neuronal dysfunction—*C9orf72* motor neurons had reduced ability to fire continuous spikes compared with control motor neurons.[132] DPR proteins have also been observed in iPSC neurons, suggesting that they are also able to model this aspect of *C9orf72* pathology. Further studies with *C9orf72* iPSCs will be important to identify disease mechanisms, and to understand the selective vulnerability of motor, cortical, and other neurons to this mutation.

What is the future for research into *C9orf72* expansions?

Much about *C9orf72* expansions is still not understood (panel 2). Reliable technologies to measure repeat length in blood, and precise data for risk and penetrance, will be important for future counselling and genetic testing, which is an area likely to expand in view of increasing awareness in the clinical community. Detailed neuroimaging and systems neuroscience studies of *C9orf72* expansions will help us to understand the cortico-thalamo-cerebellar network that seems to underlie the disorder. Novel in-vitro and in-vivo models will be essential to answer crucial questions about mechanisms of neurodegeneration, and will form the basis for the development of therapeutic options for patients with *C9orf72* expansions. However, no biomarkers of *C9orf72* expansions that might be useful in clinical trials have been validated. The results of one study have shown the presence of the GP DPR protein in CSF, but further work on this potential biomarker is needed.[139] Presymptomatic cohort studies, such as the multicentre Genetic FTD Initiative,[91,140] will be helpful to understand penetrance and in the development of biomarkers of both disease onset and progression, which will be essential for future trials aimed at treatment of people with *C9orf72* expansions.[141]

Contributors

JDR, AMI, SMi, SMe, TL, and SW did the literature review, and drafted the initial version and figures. All authors contributed to reviewing and editing the manuscript, and JDR did the final editing.

Declaration of interests

We declare no competing interests.

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