

# The clinical and pathological phenotype of C9ORF72 hexanucleotide repeat expansions

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There is increasing evidence that frontotemporal dementia and amyotrophic lateral sclerosis are part of a disease continuum. Recently, a hexanucleotide repeat expansion in *C9orf72* was identified as a major cause of both sporadic and familial frontotemporal dementia and amyotrophic lateral sclerosis. The aim of this study was to investigate clinical and neuropathological characteristics of hexanucleotide repeat expansions in *C9orf72* in a large cohort of Dutch patients with frontotemporal dementia. Repeat expansions were successfully determined in a cohort of 353 patients with sporadic or familial frontotemporal dementia with or without amyotrophic lateral sclerosis, and 522 neurologically normal controls. Immunohistochemistry was performed in a series of 10 brains from patients carrying expanded repeats using a panel of antibodies. In addition, the presence of RNA containing GGGGCC repeats in paraffin-embedded sections of post-mortem brain tissue was investigated using fluorescence

*in situ* hybridization with a locked nucleic acid probe targeting the GGGGCC repeat. Hexanucleotide repeat expansions in *C9orf72* were found in 37 patients with familial (28.7%) and five with sporadic frontotemporal dementia (2.2%). The mean age at onset was  $56.9 \pm 8.3$  years (range 39–76), and disease duration  $7.6 \pm 4.6$  years (range 1–22). The clinical phenotype of these patients varied between the behavioural variant of frontotemporal dementia ( $n = 34$ ) and primary progressive aphasia ( $n = 8$ ), with concomitant amyotrophic lateral sclerosis in seven patients. Predominant temporal atrophy on neuroimaging was present in 13 of 32 patients. Pathological examination of the 10 brains from patients carrying expanded repeats revealed frontotemporal lobar degeneration with neuronal transactive response DNA binding protein-positive inclusions of variable type, size and morphology in all brains. Fluorescence *in situ* hybridization analysis of brain material from patients with the repeat expansion, a microtubule-associated protein tau or a progranulin mutation, and controls did not show RNA-positive inclusions specific for brains with the GGGGCC repeat expansion. The hexanucleotide repeat expansion in *C9orf72* is an important cause of frontotemporal dementia with and without amyotrophic lateral sclerosis, and is sometimes associated with primary progressive aphasia. Neuropathological hallmarks include neuronal and glial inclusions, and dystrophic neurites containing transactive response DNA binding protein. Future studies are needed to explain the wide variation in clinical presentation.

**Keywords:** frontotemporal dementia; frontotemporal lobar degeneration; amyotrophic lateral sclerosis; *C9orf72* repeat expansion

**Abbreviations:** ALS = amyotrophic lateral sclerosis; FTD = frontotemporal dementia; FTL D = frontotemporal lobar degeneration; *GRN* = progranulin; *MAPT* = microtubule-associated protein tau; PPA = primary progressive aphasia; TDP = transactive response DNA binding protein; TDP-43 = transactive response DNA binding protein of 43 kDa

## Introduction

Frontotemporal dementia (FTD) is the second most common type of presenile dementia and is characterized by behavioural changes, executive and language dysfunctions due to neurodegeneration of the frontal and temporal cortex (Ratnavalli *et al.*, 2002; Seelaar *et al.*, 2011). Amyotrophic lateral sclerosis (ALS) is the most common type of motor neuron disease, characterized by rapidly progressive paralysis due to degeneration of upper and lower motor neurons leading to death within a few years (Rowland and Shneider, 2001). There is increasing clinical, pathological and genetic evidence for the hypothesis that FTD and ALS are part of a disease continuum. First of all, patients with FTD frequently develop symptoms of motor neuron disease, and cognitive dysfunction is often seen in patients with ALS (Lomen-Hoerth *et al.*, 2002). Secondly, the transactive response DNA binding protein of 43 kDa (TDP-43), an RNA binding protein, is the major pathological protein in FTD and ALS, with neuronal and glial TDP-43-positive inclusions in neocortex, basal ganglia and/or spinal cord (Neumann *et al.*, 2006; Cairns *et al.*, 2007). Thirdly, the two disorders have been shown to share genetic aetiology, apart from the genetic defects distinctive for each. Microtubule-associated protein tau (*MAPT*) and progranulin (*GRN*) mutations are exclusively associated with FTD; the same is true for superoxide dismutase 1 and optineurin mutations in ALS, but fused in sarcoma, valosin-containing protein and TDP mutations are also occasionally found in patients with FTD (Rosen, 1993; Majoor-Krakauer *et al.*, 1994; Hutton *et al.*, 1998; Baker *et al.*, 2006; Cruts *et al.*, 2006; Gros-Louis *et al.*, 2006; van Swieten and Spillantini, 2007; Seelaar *et al.*, 2008; Sreedharan *et al.*, 2008; Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Johnson *et al.*, 2010; Maruyama *et al.*, 2010). Families in which affected members present with FTD, ALS or both have shown significant linkage to chromosome 9p21.3 (Morita *et al.*, 2006; Vance *et al.*, 2006; Valdmanis *et al.*, 2007; Le Ber *et al.*, 2009;

Boxer *et al.*, 2011; Pearson *et al.*, 2011). Moreover, genome-wide association studies of both ALS and FTD have shown a significant association with the same chromosomal locus (van Es *et al.*, 2009; Laaksovirta *et al.*, 2010; Van Deerlin *et al.*, 2010). These findings indicate that this locus has a major genetic contribution to FTD and ALS. The associated risk haplotype appears to be the same for most chromosome 9p-linked families of European ancestry, suggesting a common founder (Mok *et al.*, 2011).

In September 2011, we and others simultaneously identified a (GGGGCC)<sub>n</sub> repeat expansion in a non-coding region of *C9orf72* on chromosome 9 in FTD and ALS (Dejesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). Pathogenic expanded repeats were found in 30–50% of cases with familial ALS and FTD, and in 4–10% of sporadic cases (Dejesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011).

Quantitative messenger RNA analysis has shown that the presence of the expanded repeats leads to reduced expression of one of the transcripts of *C9orf72* encoding a protein with an unknown function, suggesting a (partial) loss-of-function disease mechanism (Dejesus-Hernandez *et al.*, 2011). However, a toxic gain-of-function of abnormal messenger RNA has been hypothesized as well, based on the discovery of multiple nuclear RNA foci in brain tissue from patients carrying the expanded repeats using fluorescence *in situ* hybridization experiments with a probe targeting the GGGGCC repeat (Dejesus-Hernandez *et al.*, 2011).

As the clinical and pathological phenotype has been studied in few families with FTD + ALS so far, it is important to investigate the phenotypical variation of the repeat expansion in more detail in a larger cohort.

In the present study, we investigated the clinical and pathological characteristics of the GGGGCC hexanucleotide repeat expansion in *C9orf72* in a large Dutch cohort of patients with familial and sporadic FTD with and without ALS.

## Patients and methods

### Patients and controls

The Dutch FTD series comprises 458 patients with FTD, ascertained in an on-going genetic–epidemiological study conducted in The Netherlands since 1994, including patients that were referred to Neurology departments of the Erasmus Medical Centre or the VU University Medical Centre, or that were ascertained by research physicians visiting nursing homes and psychogeriatric hospitals. The diagnosis of FTD was based on international consensus criteria (Neary *et al.*, 1998), and concomitant ALS was diagnosed when patients also met El Escorial criteria (Brooks, 1994). Pathological confirmation of frontotemporal lobar degeneration (FTLD) was obtained in 94 patients (Mackenzie *et al.*, 2011).

The study was approved by the Medical Ethical Committee of the Erasmus Medical Centre and VU University Medical Centre. Following receipt of informed consent, DNA samples were obtained from each patient.

We excluded all patients with *MAPT* or *GRN* mutations (46 and 30 patients, respectively), or with tau-positive FTLD (19 patients). The remaining cohort to be screened for the repeat expansion in *C9orf72* consisted of 363 patients with FTD, including 38 patients with concomitant ALS. The mean age at onset was  $58.0 \pm 8.3$  years (range 28–76). The mean age at death in patients that died during follow-up ( $n = 208$ ) was  $66.3 \pm 9.7$  years (range 35–89), with mean disease duration of  $8.2 \pm 4.4$  years (range 1–23) (Table 1). The most common clinical presentation was the behavioural variant of FTD ( $n = 262$ ), followed by primary progressive aphasia (PPA) ( $n = 101$ ). Family history was positive for dementia ( $n = 130$ ), ALS ( $n = 25$ ) or Parkinson's disease ( $n = 19$ ) in at least one first-degree relative in 133 patients from 120 families. In two families, a relative with a pure ALS presentation was also genotyped.

Our control group consisted of 564 neurologically normal subjects (269 males and 295 females) from the Longitudinal Aging Study

Amsterdam (LASA, <http://www.lasa-vu.nl/>). The mean age at clinical examination for this group was  $67.8 \pm 6.0$  years (range 60–81).

### Clinical data

Detailed clinical history and family history were obtained for all patients by interviewing relatives and collecting data from medical records. We carried out a neurological examination of all patients and, when possible, patients underwent neuropsychological evaluation and neuroimaging (MRI or CT).

Neuropsychological evaluation consisted of tests for language (e.g. Boston Naming Test, Semantic Association Test, word fluency), memory (e.g. Rey Auditory Verbal Learning Test, Visual Association Test), attention and concentration and executive functions (e.g. Trail Making Test, Stroop colour-word test, modified Wisconsin Card Sorting Test, Similarities and Proverbs of the Wechsler Adult Intelligence Scale) and visuospatial abilities (e.g. Clock drawing, Block Design of the Wechsler Adult Intelligence Scale). The presence and severity of frontal, temporal, parietal, occipital and cerebellar atrophy were reviewed by a neurologist (J.C.v.S.) and a radiologist (M.S.). Patients with signs suggestive of ALS, such as muscle weakness, atrophy or fasciculations, underwent EMG. The age at onset was defined as the moment partners or other relatives noticed the first symptoms attributable to the disease.

Three classes of family history were distinguished: (i) autosomal dominant, patients with at least two first-degree relatives with dementia or ALS; (ii) patients with only a single affected first-degree relative with dementia or ALS; and (iii) patients without affected relatives or an unknown family history.

### Genotyping methods

For the repeat-primed polymerase chain reaction 50 ng of genomic DNA from each patient, was mixed with FastStart Taq DNA polymerase PCR buffer (Roche Applied Science), 7-deaza-dGTP (New England Biolabs), Q-Solution (Qiagen Inc.), dimethylsulphoxide Hybri-Max (Sigma-Aldrich),  $MgCl_2$  (Roche Applied Science), reverse primer consisting of four GGGGCC repeats with an anchor tail, 6FAM<sup>TM</sup>-fluorescent labelled forward primer located 128-bp telomeric to the repeat sequence, and an anchor primer corresponding to the anchor tail of the reverse primer, as described in our previous article (Renton *et al.*, 2011). Primer sequences are available upon request (Warner *et al.*, 1996; Kobayashi *et al.*, 2011). Fragment length analysis was performed on an ABI 3730xl genetic analyzer (Applied Biosystems), and data analysed using Peak Scanner software version 1.0 (Applied Biosystems). Repeat expansions produce a characteristic saw tooth pattern with a 6-bp periodicity (Supplementary Fig. 1). We obtained hexanucleotide repeat lengths based on the repeat-primed polymerase chain reaction assay by successful genotyping in 353 cases and 522 controls (94.4% of the total cohort).

To note, the repeat-primed polymerase chain reaction assay used for these experiments does not determine the actual number of repeats in a large pathogenic expansion. This technique only allows for testing whether a given sample carries a large pathogenic expansion or not. A cut-off value of 30 repeats was used to define expanded repeats, as previously described (Renton *et al.*, 2011).

### Pathological examination

Brain autopsy was carried out within 4 h of death according to the Legal and Ethical Code of Conduct of the Netherlands Brain Bank. Tissue blocks taken from all cortical areas, hippocampus, amygdala,

**Table 1** Demographic features of the Dutch FTD cohort

	FTD cohort (n = 363)
Female (%)	178 (49.0)
Age at onset, years (range)	$58.0 \pm 8.3$ (28–76)
Age at death ( $n = 208$ ), years (range)	$66.3 \pm 9.7$ (35–89)
Disease duration ( $n = 208$ ), years (range)	$8.2 \pm 4.4$ (1–23)
FTD subtype	
bvFTD (%)	262 (72.2)
PPA (%)	101 (27.8)
ALS	38 (10.5)
Family history	
Positive for dementia (% , no. of families)	130 (35.8, 117)
Positive for ALS (% , no. of families)	25 (6.9, 17)
Positive for PD (% , no. of families)	19 (5.2, 16)
Negative (%)	230 (63.4)
Neuropathological examination ( $n = 51$ )	
FTLD-TDP	45 (88.2)
FTLD-FUS	4 (7.8)
FTLD-ni	1 (2.0)
FTLD (subtype unknown)	1 (2.0)

bvFTD = behavioural variant of FTD; FUS = fused in sarcoma; ni = no inclusions.

basal ganglia, substantia nigra, pons, medulla oblongata, cerebellum and cervical spinal cord were embedded in paraffin blocks, and underwent routine staining with haematoxylin–eosin, Bodian, methenamine–silver and Congo red. Tissue blocks were taken from the right hemisphere in each case. Immunohistochemistry was performed using primary antibodies against hyperphosphorylated tau (AT8, Innogenetics; 1:40), ubiquitin (anti-ubiquitin, Dako; 1:500, following 80°C antigen retrieval),  $\beta$ -amyloid protein (anti-beta amyloid, Dako; 1:100, following formic acid pretreatment),  $\alpha$ -synuclein (anti- $\alpha$ -synuclein, Zymed Laboratories; undiluted, following formic acid pretreatment), p62 (BD Biosciences Pharmingen; 1:200, following 80°C antigen retrieval), TDP-43 (Biotech; 1:100, following pressure cooking), TDP-43 phosphorylated at serine 409/410 (Cosmo Bio; 1:8000), fused in sarcoma (Sigma-Aldrich anti-fused in sarcoma; 1:25–200 with initial overnight incubation at room temperature, following pressure cooking) and *C9orf72* (GeneTex; 1:200) and stained as previously described (Seelaar *et al.*, 2007). Primary antibodies were incubated overnight at 4°C. Endogenous peroxidase activity was inhibited by incubation in phosphate buffered saline–hydrogen peroxide–sodium azide solution (100 ml 0.1 M phosphate-buffered saline + 2 ml 30% H<sub>2</sub>O<sub>2</sub> + 1 ml sodium azide) for 30 min. The Histostain-Plus broad-spectrum kit DAB (Zymed) was used, and slides were counterstained with Mayer’s haematoxylin and mounted in Entellan<sup>®</sup>. The pathological diagnosis was made by a neuropathologist (A.J.M.R.).

Brain autopsy performed in 51 out of the total cohort of 363 patients revealed TDP-43-positive pathology (FTLD-TDP) in 45 patients, FTLD with fused in sarcoma-positive pathology in five, FTLD with no inclusions in one and FTLD (subtype unknown) in one.

The pattern of FTLD-TDP pathology was classified into the four following subtypes: type A is characterized by numerous short dystrophic neurites and crescentic or oval neuronal cytoplasmic inclusions, concentrated primarily in neocortical layer 2. Moderate numbers of lentiform neuronal intranuclear inclusions are also a common but inconsistent feature of this subtype; type B by moderate numbers of neuronal cytoplasmic inclusions, throughout all cortical layers, but very few dystrophic neurites; type C by a predominance of elongated dystrophic neurites in upper cortical layers, with very few neuronal cytoplasmic inclusions; and type D by numerous short dystrophic neurites and frequent lentiform neuronal intranuclear inclusions (Mackenzie *et al.*, 2011).

## Fluorescence *in situ* hybridization

The hypothesis of a toxic RNA gain-of-function mechanism for FTD/ALS suggests that RNA containing expanded non-coding hexanucleotide repeats accumulates in affected cells. To test this hypothesis, we examined paraffin-embedded sections of post-mortem temporal cortex and hippocampal tissue for the presence of RNA containing GGGGCC repeats using fluorescence *in situ* hybridization. For RNA-fluorescence *in situ* hybridization, brain sections were hybridized either with an oligonucleotide probe (GGCCCC)<sub>3</sub> 5' TYE563 or a CCCC GGCCCC 5' TYE563 labelled locked nucleic acid oligonucleotide probe (both Exicon), which differs from the method used by DeJesus-Hernandez *et al.* (2011). After the RNA-fluorescence *in situ* hybridization protocol the slides were incubated with Hoechst stain in phosphate-buffered saline (1:15 000) and washed two times for 5 min with phosphate-buffered saline, followed by one wash in de-ionized water, before mounting in Mowiol<sup>®</sup>. Slides were examined using a confocal fluorescence microscope (Leica).

## Statistical analysis

Fisher’s exact test was used to test for association between the presence of *C9orf72* repeat expansion and both familial and sporadic FTD using the PLINK v1.07 toolset. Since presence of individuals from the same family could bias our association results, only one affected individual was included per family in this analysis.

Independent samples *t*-tests to compare continuous variables between patients with the *C9orf72* repeat expansion and patients with *MAPT* or *GRN* mutations were performed using SPSS 17.0 for windows (SPSS). A significance level of  $P < 0.05$  was used.

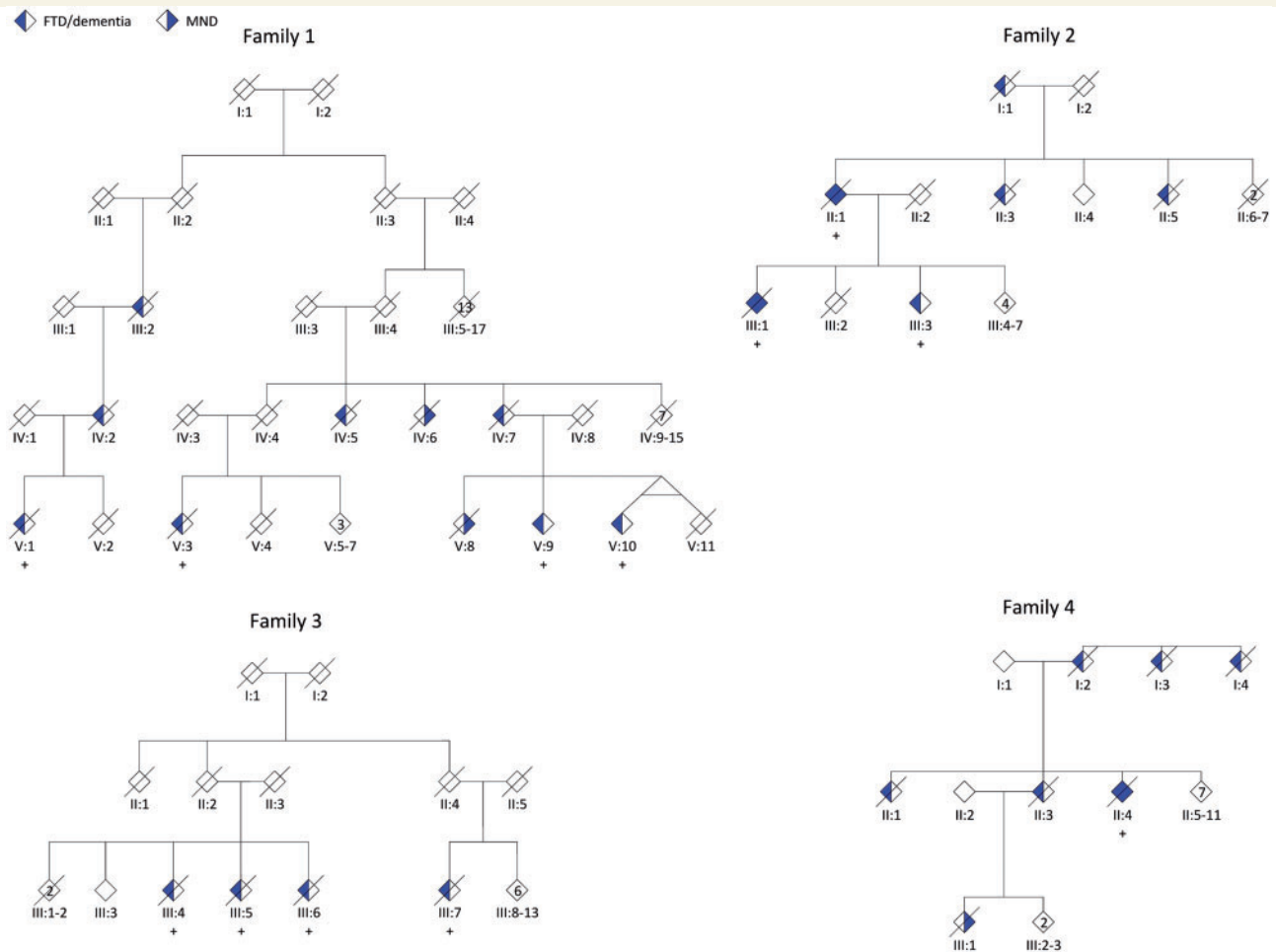
## Results

### Genotyping results

A total of 353 cases with FTD and 522 controls were successfully genotyped with the repeat-primed polymerase chain reaction assay. Histograms of repeat lengths based on the repeat-primed polymerase chain reaction assay are shown for both cases and controls (Supplementary Fig. 2). The average repeat length in the control population was  $9.1 \pm 6.8$  (range 2–35 repeats) and for the cases with FTD, the average repeat length was  $13.9 \pm 14.0$  (range 1–64 repeats). A total of 42 cases from our cohort (11.9%) and three controls (0.6%) carried the expansion (Fisher’s test  $P$ -value =  $4.39 \times 10^{-12}$ ; odds ratio = 19.22, 95% confidence interval = 5.89–62.66 after removal of cases belonging to the same family). Thirty-seven patients with FTD with the expansion had a positive family history (28.7% of genotyped patients with FTD with positive family history), with an autosomal dominant mode of inheritance in 25 patients (19 families, four of which are shown in Fig. 1), including four families where reduced penetrance was observed (Family 3 in Fig. 1). Obligate carriers in two of the latter families died after the age of 70 without any signs of dementia or ALS. The remaining 12 patients with a positive family history had only one first-degree family member with either dementia or ALS (11 families) (Table 2). Fisher association analysis of familial FTD cases versus controls (individuals belonging to the same family were removed for this analysis) gave the following results: Fisher’s test  $P$ -value =  $1.76 \times 10^{-20}$ , odds ratio = 51.47, 95% confidence interval = 15.95–169.90. There was wide phenotypic variability within families with diagnoses of both FTD, ALS and FTD + ALS in 12 families. Furthermore three families included individuals with Parkinson’s disease, however, we cannot be certain that this is also caused by the repeat expansion, since these individuals were not genotyped.

In one family with autosomal dominant FTD + ALS the proband included in our clinical FTD cohort had a repeat length of 26 and was therefore assumed not to carry the repeat expansion. However, sequencing in family members revealed the repeat expansion in a so far unaffected person (age 42 years) and repeat length varying from 8 to 29 in affected persons. Therefore, we are uncertain about the pathogenicity of the repeats in this family, especially as it is not yet possible to determine exact repeat lengths.

Moreover, in three of the families with the repeat expansion, there was one affected individual with a repeat length of 29.



**Figure 1** Pedigrees for Families I–IV. Subjects that were included in the sequencing cohort are indicated with (+). MND = motor neuron disease; FTD = frontotemporal dementia.

The repeat expansion in *C9orf72* was found in 5 of the 224 genotyped patients with sporadic FTD (2.2%), which was not significantly different from healthy controls (Fisher's test  $P = 0.0569$ , odds ratio = 3.93, 95% confidence interval = 0.93–16.53).

Of the remaining 311 patients in the cohort without the repeat expansion, 92 had a positive family history for dementia (89 families), with ALS in six families. Moreover, 31 of the patients with FTD without the repeat expansion had concomitant ALS.

In an effort to investigate whether the expansion carriers in our cohort carry the recently identified risk haplotype on chromosome 9 (Mok *et al.*, 2011), genotyping data from a parallel project in our laboratory was extracted for 20 of the 42 expansion carriers (Fig. 2). Genotypes of 12 of these samples (60.0%) were concordant with the reported risk haplotype. Interestingly, all other samples shared the same core risk haplotype, differing from it only in the most distal positions. These results suggest that all Dutch *C9orf72* carriers derive from a common mutated ancestor.

## Clinical features

The mean age at onset in the 42 patients with FTD with the repeat expansion was  $56.9 \pm 8.3$  years (range 39–76), mean age

at death ( $n = 31$ ) was  $64.7 \pm 8.6$  years (range 42–78) and mean disease duration from onset till death was  $7.6 \pm 4.6$  years (range 1–22). Behavioural variant FTD was the initial clinical presentation in 34 patients (apathy in 18, disinhibition in 11, obsessive–compulsive behaviour in five), and PPA in eight patients (Fig. 3). Concomitant ALS was present in seven patients (bulbar onset in five, limb onset in two). Furthermore, we found the repeat expansion in two relatives of patients with FTD with pure limb onset ALS. Mean score at the Mini-Mental State Examination was  $25.9 \pm 3.4$  ( $n = 19$ , range 17–30). Memory complaints were reported in 21 patients at clinical presentation. Six patients showed signs of Parkinsonism and apraxia was present in seven patients. Visual or auditory hallucinations were reported in two patients and delusions in none. Mean duration of follow-up from disease onset in patients with the repeat expansion was  $5.2 \pm 3.3$  years (range 0.8–13.4).

Of the eight patients with PPA, two showed fluent speech, anomia and single-word comprehension deficits at neuropsychological evaluation compatible with the diagnosis semantic dementia, supported by atrophy of the anterior temporal lobes (Fig. 4). Classification into one of the PPA variants was not possible in the remaining six patients due to lack of information. Four of them

Table 2 Clinical and pathological features of patients with FTD with repeat expansion

	No. of affected (confirmed expansion)	No. of families	Age at onset (range)	Age at death (range)	Disease duration (range)	FTD subtype (no. of patients)	Imaging <sup>a</sup> (no. of patients)	Pathology (no. of patients)
Autosomal dominant								
Family 1	10 (3)	1	60.8 (55–67)	70.0 (64–75)	10.0 (9–12)	bvFTD (8), ALS (2)	T (1), FT (2), G (1)	na
Family 2	6 (2)	1	59.2 (39–80)	70.2 (59–84)	7.0 (1–17)	bvFTD (4), bvFTD + ALS (2)	N (1), T (1), FT (1)	FTLD-TDP (1)
Family 3	4 (3)	1	54.1 (45–62)	59.2 (52–64)	8.0 (6–9)	bvFTD (4)	N (1), T (2), G (1)	FTLD-TDP (2)
Family 4	7 (1)	1	65.0 (64–67)	69.3 (67–73)	4.0 (1–8)	bvFTD (5), ALS (1), FTD + ALS (1)	T (1)	na
Remaining autosomal dominant cases	18 (18)	15	57.1 (39–67)	65.4 (46–75)	7.2 (1–18)	bvFTD (13), PPA (1), ALS (2), bvFTD + ALS (1), PPA + ALS (1)	F (1), T (2), FT (5), N (1), G (1)	FTLD-TDP (6)
Cases with only one affected family member	12 (12)	11	54.2 (39–76)	62.7 (42–78)	8.2 (2–22)	bvFTD (6), PPA (3), bvFTD + ALS (3)	N (2), F (2), T (2), FT (1), G (1)	FTLD-TDP (2)
No family history	5 (5)	5	61.3 (55–70)	67.9 (64–72)	9.1 (6–11)	bvFTD (2), PPA (2), bvFTD + ALS (1)	T (4), F (1)	FTLD-TDP (2)

<sup>a</sup>Area of most prominent atrophy is indicated.

bvFTD = behavioural variant of frontotemporal dementia; F = frontal; G = generalized; FT = frontotemporal; N = no atrophy; na = not available; T = temporal.

had fluent speech, anomia and impaired language comprehension according to their history, but extensive neuropsychological evaluation was not available or possible at the time of out-clinic visits. The other two had non-fluent speech with anomia and comprehension deficits in one.

All patients with behavioural variant FTD who underwent extensive neuropsychological evaluation had executive dysfunctions, and 10 of them had severe language deficits on initial presentation.

Neuroimaging was available for 32 patients with the repeat expansion. The pattern of cerebral atrophy was predominantly anterior temporal in 13 patients, frontal in four and frontotemporal in seven patients. In all patients with PPA, atrophy was most prominent in the temporal cortex. In four patients, the atrophy was generalized, and four patients (including a patient with pure ALS) had no atrophy. Atrophy was extended into the parietal cortex in 10 patients, and into the occipital cortex in one. Mild cerebellar atrophy was found in eight patients.

## Neuropathological findings

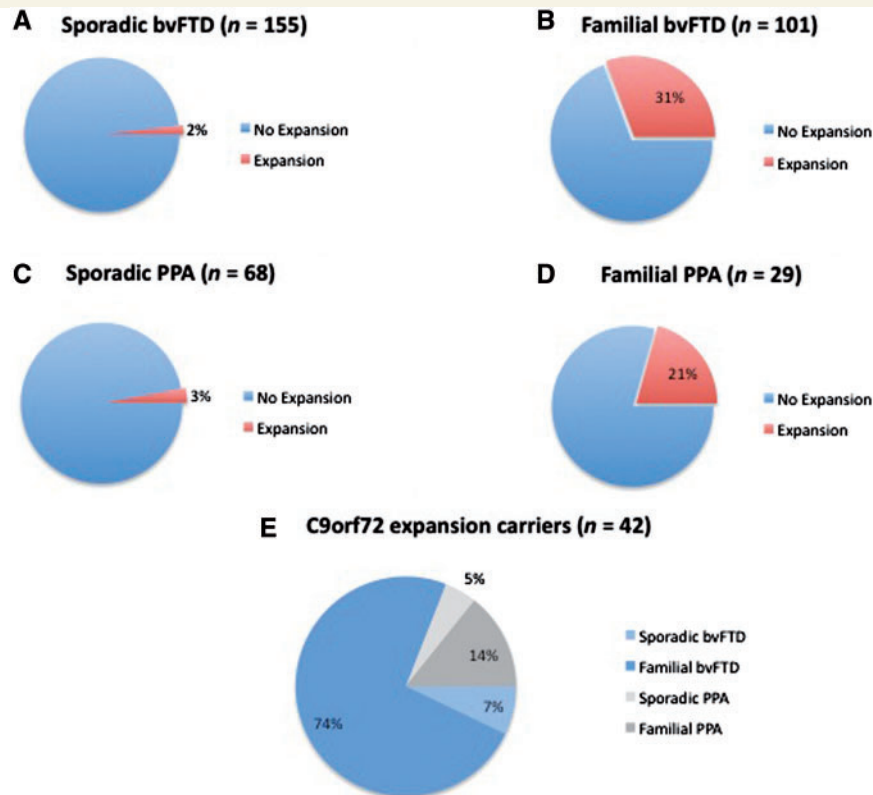
Brain autopsy was carried out by The Netherlands Brain Bank in 10 patients carrying the pathogenic repeat expansion and in the patient with a repeat length of 26 with an unaffected family member with the expansion (Table 3). The brain weight was reduced (mean 1112 g, range 886–1297). Macroscopy showed moderate to severe frontal and temporal atrophy in all except one brain. Hypopigmentation of the substantia nigra was found in three brains.

Routine staining showed variable neuronal loss in the frontal and/or temporal cortex in all, except for two cases with FTD + ALS. In the PPA case, atrophy and neuronal loss was most severe in the temporal cortex. Mild neuronal loss in the substantia nigra was seen in seven cases and in the caudate nucleus and putamen in two.

Immunohistochemistry with ubiquitin, p62 and TDP-43 antibodies revealed TDP-43 type B pathology in all brains (Mackenzie *et al.*, 2011). Many neuronal cytoplasmic inclusions of variable size (round, crescent, granular) and morphology (diffuse, dense) were seen, most abundant in the dentate gyrus of the hippocampus (Fig. 5A), in superficial and deeper layers of the temporal, frontal and parietal cortex (Fig. 5B), and with less density in the basal ganglia. Irregular-shaped aggregates were seen in many pyramidal cells of cornu ammonis 3 and 4. Many short, thin or swollen dystrophic neurites were seen in cortical areas in most cases, with the presence of long dystrophic neurites in the parietal cortex of three brains, and in the temporal cortex in only the PPA case (Fig. 5C). Three brains showed a variable number of neuronal intranuclear inclusions in neocortex or basal ganglia (Fig. 5D). A few irregular shaped or skein-like TDP-43- and p62-positive inclusions were found in the substantia nigra (Fig. 5E) and brainstem (Fig. 5F) of four brains. Small dense neuronal p62-positive inclusions and short neurites in the granular layer of the cerebellum were seen in 9 out of 11 brains (Fig. 5G). Some p62- and TDP-43-positive glial inclusions (oligodendroglia like) were found in the subcortical white matter in a number of brains (Fig. 5H). These glial inclusions did not stain with ubiquitin antibody.

SNP ID	Position	Risk allele	Freq CEU	D95.2993	D96.0248	D98.8895	D98.8369	07D01816	D96.1388	D95.3021	D96.2281	D99.3488	D99.4036	D01.2972	D99.2671	06D01622	06D05358	07D01145	07D01783	07D01836	08D01457	06D03315	05D00499	
rs1622723	27468052	C	0.708	C/C	C/C	T/C	T/C	C/C	C/C	C/C	C/C	T/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	T/C	T/C	C/C	C/C
rs4879515	27472235	T	0.39	C/T	C/T	T/T	C/T	T/T	C/T	T/T	C/T	C/T	C/T	C/T	C/T	C/T	T/T	C/T	C/T	C/T	T/T	T/T	C/T	C/T
rs868856	27479251	T	0.275	C/T	C/T	C/T	T/T	T/T	C/T	C/T	C/T	T/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T
rs7046653	27480967	A	0.317	G/A	G/A	G/A	A/A	A/A	G/A	G/A	A/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
rs1977661	27492986	C	0.892	C/C	C/C	A/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	A/C	C/C
rs903603	27519316	C	0.475	T/C	T/C	T/C	C/C	C/C	T/C	T/C	C/C	C/C	T/C	T/C	T/C	T/C	C/C	T/C	T/C	T/C	C/C	T/C	T/C	T/C
rs10812610	27523984	C	0.433	A/C	A/C	A/C	C/C	C/C	A/C	A/C	C/C	C/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C
rs2814707	27526397	A	0.192	G/A	G/A	G/A	G/A	A/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
rs3849942	27533281	A	0.183	G/A	G/A	G/A	A/A	A/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
rs10122902	27546780	G	0.833	G/G	G/G	G/G	A/G	G/G	G/G	G/G	G/G	A/G	G/G	G/G	G/G	G/G	A/G	G/G	G/G	G/G	A/G	G/G	G/G	
rs10757665	27547919	T	0.733	C/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	C/T	T/T	T/T	T/T	T/T	T/T	T/T	C/T	T/T	T/T	T/T	T/T
rs1565948	27549733	G	0.467	A/G	G/G	G/G	A/G	G/G	G/G	A/G	A/G	A/G	G/G	G/G	G/G	A/G	G/G	A/G	G/G	A/G	A/G	A/G	G/G	G/G
rs774359	27551049	C	0.225	T/C	T/C	T/C	T/C	C/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C
rs2282241	27562255	G	0.533	G/G	T/G	T/G	T/G	G/G	T/G	T/G	T/G	T/G	G/G	T/G	T/G	T/G	T/G	T/G	T/G	T/G	T/G	T/G	T/G	T/G
rs1948522	27565785	C	0.767	C/C	C/C	C/C	T/C	C/C	C/C	T/C	T/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	T/C	T/C	C/C	C/C
rs1982915	27569560	G	0.5	A/G	A/A	A/G	G/G	G/G	A/G	A/G	G/G	G/G	A/G	A/G	A/G	G/G	G/G	G/G	A/G	A/G	A/G	G/G	A/G	A/G
rs2453556	27576162	G	0.325	A/G	A/A	A/G	G/G	G/G	A/G	A/A	A/G	G/G	A/G	A/G	A/G	A/G	G/G	A/G	G/G	A/G	A/G	G/G	A/G	A/G
rs702231	27578731	A	0.783	C/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	C/A	A/A	A/A	A/A	C/A	A/A	C/A	A/A	C/A	A/A	A/A	A/A	A/A
rs696826	27579657	G	0.892	A/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	A/G	G/G	G/G	G/G	G/G	A/G	G/G	G/G	G/G
rs2477518	27589746	T	0.725	C/T	T/T	T/T	C/C	C/C	C/T	T/T	T/T	C/C	T/T	C/C	C/T	C/T	C/C	C/T	C/T	C/T	C/C	C/T	T/T	T/T

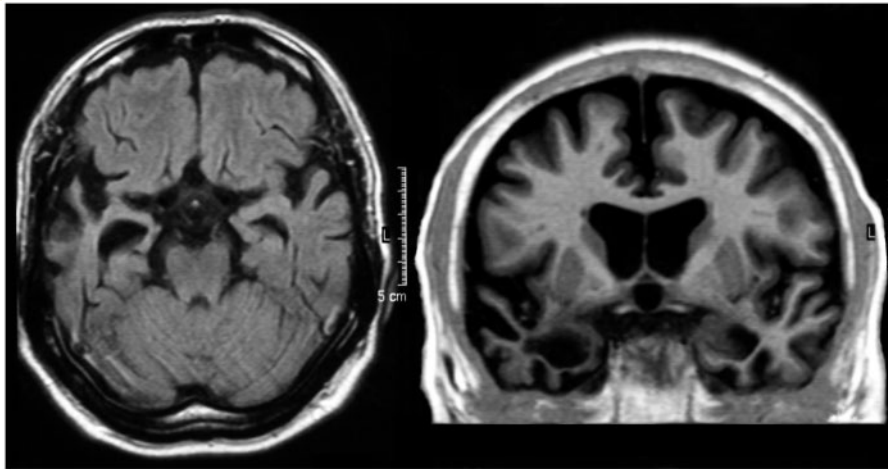
**Figure 2** Genotyping data for 20 expansion carriers for which genome-wide association data were available. Those genotypes that are concordant with the allele in the risk haplotype according to Mok *et al.* (2011) are shaded in blue. Individuals 06D03315, 08D01457, D98.8895, D952993 and 07D01783 did not present with family history of FTD or ALS. Freq CEU = Frequency in CEU population (Ceph Europeans from Utah); SNP = single nucleotide polymorphism.



**Figure 3** Clinical features of C9orf72 expansion carriers. (A–D) display C9orf72 expansion distribution depending on the clinical phenotype (behavioural variant FTD or PPA) and family history for FTD or ALS. (E) shows the clinical distribution of all 42 expansion carriers.

In one brain, many p62-, and TDP-43-positive glial inclusions of astrocytic nature were seen in the parietal and temporal cortex and the neostriatum (Fig. 5I). TDP pathology was not more abundant in areas of atrophy. The p62 staining of inclusions was more intense than TDP-43 staining in all cases. Immunohistochemistry with AT8

and  $\beta$ -amyloid antibodies showed abundant neurofibrillary tangles and  $\beta$ -amyloid plaques in the temporal cortex in two brains (Braak stage 2C) (Thal *et al.*, 2002). Corticospinal tract degeneration was found in three brains. Immunohistochemistry with C9orf72 antibody shows that C9orf72 is a largely cytoplasmatic protein in neurons.



**Figure 4** MRI scan of a patient with semantic dementia showing severe temporal atrophy. L = left.

**Table 3** Neuropathological findings in patients with a repeat expansion in *C9orf72*

Case	1 (Family 3 III:5)	2 (Family 3 III:7)	3	4	5	6	7	8	9	10	11 <sup>e</sup>
Clinical presentation	bvFTD	bvFTD	bvFTD	bvFTD + ALS	bvFTD	bvFTD	PPA	bvFTD	bvFTD	bvFTD + ALS	bvFTD + ALS
Brain weight (g)	1065	1297	1220	1096	1095	1150	1245	1146	886	958	1076
Atrophy	F, T, P, SN	F, T,	F, T, SN	Normal	F, T, P	F, T, SN	T	F, T, P	F, T	F, T, P	F
Neuronal loss											
Frontal	+	–	–	–	+	+	–	+	+	+	–
Temporal	++	++	++	+	++	++	++	++	+	+	++
Spinal cord	–	–	–	+	–	–	+	–	na	na	++
Substantia nigra	+	–	+	++	+	+	–	–	–	+	++
IHC: TDP43/p62 <sup>a</sup>											
Frontal	++	++	++ <sup>c</sup>	<sup>d</sup>	+	+	–	++	+	–	+
Temporal	++	++	++	+	+	++	<sup>b</sup>	++	+	++	++
Parietal	<sup>b,c</sup>	<sup>b</sup>	+	<sup>d</sup>	+	++	<sup>b</sup>	+	+	+	+
Hippocampus	+	++	++	+	++	++	++	++	++	++	++ <sup>c</sup>
Basal ganglia	+	+	++ <sup>c</sup>	<sup>d</sup>	+	na	+	++	–	–	<sup>c</sup>
Cerebellum	++	++	+	<sup>d</sup>	+	na	–	+	++	–	++
Brainstem	+	–	na	+	+	–	–	–	+	+	–
Spinal cord	na	na	na	+	na	–	na	–	na	na	+

a p62- or TDP43-positive neuronal cytoplasmic inclusions and/or dystrophic neurites.

b Long dystrophic neurites.

c Neuronal intranuclear inclusions.

d Glial inclusions.

e The repeat expansion was not confirmed in this case, but repeat length was 26, and a so far unaffected relative did carry the repeat expansion.

F = frontal; H = hippocampus; IHC: TDP43/p62 = immunohistochemistry with p62 and/or TDP43 antibodies; P = parietal; T = temporal; SN = substantia nigra; – = none; + = mild; ++ = moderate; +++ = severe; na = not available.

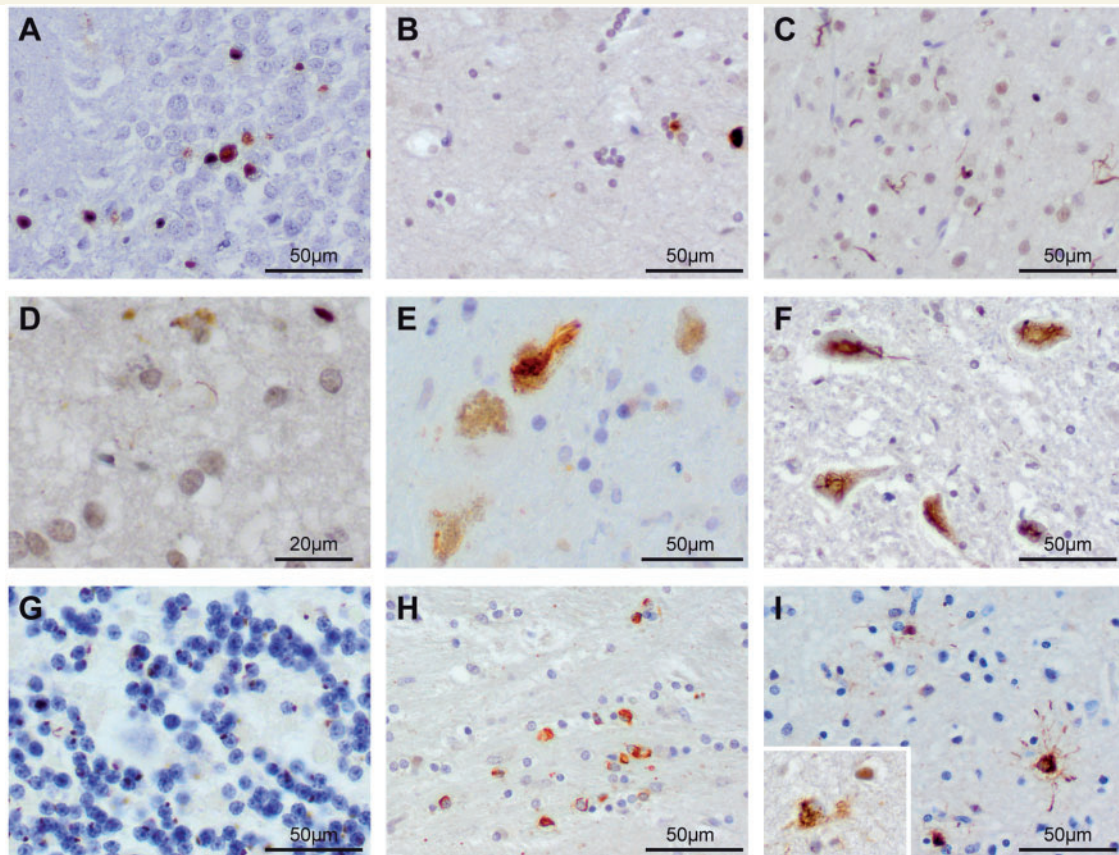
Immunostaining with the antibody against *C9orf72* protein showed a granular staining of the cytoplasm into the dendritic arborizations of neurons in cornu ammonis 3 and 4, but this was observed in FTD both with and without the repeat expansion.

Of another five brains from patients with the pathogenic repeat expansion from other academic centres, the pathological diagnosis was FTLD with ubiquitin pathology. However, brain tissue was not available for extensive assessment using TDP-43 and p62 antibodies.

## Fluorescence *in situ* hybridization

The RNA-fluorescence *in situ* hybridization results with the locked nucleic acid probe were inconsistent, that is, we analysed post-mortem tissue of three patients with the expanded GGGGCC repeat, three patients with a *GRN* mutation, three patients with *MAPT* mutations, one patient with fragile X-associated tremor/ataxia syndrome (CGG<sub>98</sub> repeat expansion) and three non-demented controls for the presence of RNA foci. With the





**Figure 5** Immunohistochemistry with p62 and TDP-43 antibodies in brains of patients carrying the GGGGCC repeat expansion in *C9orf72*. Many dense neuronal cytoplasmic inclusions were present in the granular cells of the dentate gyrus (A). Dense or granular cytoplasmic inclusions of variable size, and short dystrophic neurites were visible in the deep and superficial layers of the frontal and temporal cortex (B). Long dystrophic neurites were seen in the cortical areas of a few brains (C). The temporal and parietal cortex showed a number of TDP-43-positive neuronal intranuclear inclusions (D). Some skein-like or filamentous TDP-43-positive inclusions were found in neurons of the substantia nigra (E) and lower motor neurons in the brainstem (F). Abundant small cytoplasmic p62-positive, TDP-43-negative inclusions and short neurites were seen in the granular layer of the cerebellum (G). Abundant p62-positive cytoplasmic glial inclusions were present in white matter of the striatum (H). Several p62-positive inclusions in the cytoplasm and dendritic processes of glial (probably astrocytic) cells in the temporal cortex of one brain from a patient with FTD + ALS (I), TDP-43 antibody also has a positive, although weaker, staining of these inclusions (inset).

locked nucleic acid probe we did find RNA-positive inclusions in brains with GGGGCC repeat expansions, but also in three cases with *MAPT* mutations and in a non-demented control. The specificity of the staining observed with the locked nucleic acid probe remains to be determined. DeJesus-Hernandez *et al.* did not test cases with *MAPT* mutations (DeJesus-Hernandez *et al.*, 2011). Using the oligonucleotide probe (GGCCCC)<sub>3</sub> 5' TYE563, no RNA foci could be detected in any of the samples studied.

### Comparison with *MAPT* and *GRN* mutation carriers

Whereas the *MAPT* and *GRN* mutation carriers in the Dutch cohort came from 12 and 6 large families, respectively; the carriers of the repeat expansions in *C9orf72* came from 35 apparently unrelated families. There was no significant difference in age at onset, age at death or disease duration between repeat expansion

carriers and *MAPT* or *GRN* mutation carriers (Table 4). The *C9orf72* repeat expansions are associated with a wider phenotypic variability than *MAPT* and *GRN* mutations. In contrast to patients with *MAPT* and *GRN* mutations, concomitant ALS was a frequent finding in patients with the *C9orf72* repeat expansion. The frequent finding of predominant temporal atrophy is in contrast to the imaging features in *MAPT* and *GRN* mutations, where predominant frontal atrophy was more common.

### Discussion

The present study shows that the pathogenic hexanucleotide expansion in *C9orf72* is one of the most common genetic causes of familial FTD in The Netherlands, and that the repeat expansion is associated with a wide variation in clinical phenotype (behavioural variant FTD, PPA, ALS) and with predominant temporal atrophy on neuroimaging.

**Table 4** Clinical features in patients with FTD with a repeat expansion in *C9orf72* compared with patients with *MAPT* and *GRN* mutations

	Repeat expansion in <i>C9orf72</i> (n = 42)	<i>MAPT</i> mutation (n = 46)	<i>GRN</i> mutation (n = 30)
No. of families	35	12	6
Female (%)	22 (52.4)	24 (52.2)	19 (63.3)
Age at onset, years (range)	56.9 ± 8.3 (39–76)	52.3 ± 6.0 (39–65)	60.6 ± 9.3 (45–79)
Age at death, years (range)	64.7 ± 8.6 (42–78)	61.2 ± 7.7 (44–76)	69.4 ± 10.2 (52–87)
Duration of illness, years (range)	7.6 ± 4.6 (1–22)	9.2 ± 4.5 (3–20)	7.4 ± 2.8 (2–13)
Clinical subtype			
BvFTD (%)	34 (81.0)	45 (97.8)	28 (93.3)
PPA (%)	8 (19.0)	1 (2.2)	2 (6.7)
ALS (%)	7 (16.7)	0	0
Imaging <sup>a</sup>			
No atrophy (%)	3 (9.7)	0	0
Frontal (%)	4 (12.9)	23 (53.5)	23 (85.2)
Temporal (%)	13 (41.9)	15 (34.9)	1 (3.7)
Frontotemporal (%)	7 (22.6)	4 (9.3)	1 (3.7)
Generalized (%)	4 (12.9)	1 (2.3)	2 (7.4)

a Area of predominant atrophy.

BvFTD = behavioural variant of frontotemporal dementia.

The percentage of the repeat expansion in the present series of familial FTD is higher than in the study by DeJesus-Hernandez *et al.* (28.7% versus 11.7%), which can be explained by the exclusion of patients with *MAPT* and *GRN* mutations in this study (DeJesus-Hernandez *et al.*, 2011). In this regard, if the whole population of screened patients with FTD and patients with *MAPT* and *GRN* mutations or tau-positive FTLD ( $n = 448$ ) is accounted for, the *C9orf72* hexanucleotide expansion accounts for 17.8% of familial and 9.4% of total FTD in The Netherlands. Of the total population of patients with FTD in The Netherlands, 26.3% is explained by mutations in *GRN* (6.7%), *MAPT* (10.3%) and the hexanucleotide expansion in *C9orf72* (9.4%). Considering only familial FTD, we can now explain up to 53.8% cases in The Netherlands (*GRN*: 13.9%, *MAPT*: 22.1% and *C9orf72*: 17.8%).

The frequency of the repeat expansion in the present series of sporadic FTD is in line with the findings in the study by DeJesus-Hernandez *et al.* (2011). Although a larger proportion of sporadic FTD cases carry the expansion in comparison to controls, this difference was not statistically significant ( $P = 0.0569$ ). A larger cohort of sporadic patients has to be screened to unveil the role of this expansion in the sporadic form of the disease.

Genetic analysis of a subset of cases carrying this expansion showed that patients from apparently unrelated families carry the same risk haplotype, indicating that there is a common ancestor for all patients with expanded alleles of *C9orf72*. As this risk haplotype was also found in the five apparently sporadic cases, this indicates that these cases are in fact, cryptically related familial cases. The negative family history for these patients could be explained by early death of affected family members, non-paternity or a lack of medical information in previous generations. Another possible explanation of the occurrence of the repeat expansion in apparently sporadic cases in this and other studies, could be reduced penetrance of the repeat expansion in *C9orf72*

(DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). This reduced penetrance is evident in at least two of the families in which unaffected obligate carriers lived long enough to develop the disease, although the possibility of non-paternity cannot be ruled out. The reduced penetrance is in line with previous reports on chromosome 9p-linked FTD, in which the repeat expansion has yet to be confirmed (Vance *et al.*, 2006). Another explanation may be that additional genetic and/or environmental factors may determine the wide inter- and intra-familial variation in age at onset and clinical presentation in the present and other chromosome 9p-linked families (Valdmanis *et al.*, 2007; Le Ber *et al.*, 2009; Boxer *et al.*, 2011; Pearson *et al.*, 2011). If reliable measurement of the exact length of the expanded repeats becomes possible in the future, further studies are needed to investigate the correlation between age at onset and repeat expansion length, and the possibility of anticipation, as observed in other repeat expansion disorders.

The observed clinical heterogeneity within families, including behavioural variant FTD, PPA, ALS and Parkinsonism in the present study, is in line with previous observations in chromosome 9p-linked families. Concomitant ALS was a frequent finding (seven patients) in patients with FTD with the repeat expansion, and this frequency may even be an underestimation, since follow-up duration from disease onset was highly variable. PPA, defined as a prominent, isolated language deficit during the initial phase (Gorno-Tempini *et al.*, 2011), also frequently occurred in the present series (eight patients) and in a Finnish series of patients with FTD (Renton *et al.*, 2011), but not in previously reported chromosome 9p-linked families (Morita *et al.*, 2006; Valdmanis *et al.*, 2007; Le Ber *et al.*, 2009). An association between PPA and ALS is further supported by a high frequency of a language-dominant presentation in patients with FTD + ALS (Coon *et al.*, 2011), which suggests a common cortical degenerative process for the language abnormalities in PPA, and tongue

and bulbar muscle weakness in ALS. Two of the present patients with PPA with the pathogenic repeat expansion, without ALS symptoms, were classified as semantic dementia, which was supported by the presence of severe anomia and single-word comprehension deficits, and by atrophy of the anterior temporal lobes on neuroimaging (Gorno-Tempini *et al.*, 2011). The occurrence of semantic dementia was unexpected, as the association of semantic dementia and ALS has only been described in a few cases in a recent report (Kim *et al.*, 2009; Coon *et al.*, 2011). Furthermore, patients with semantic dementia usually have a negative family history, which diminishes the likelihood of a genetic factor with a dominant effect in semantic dementia (Goldman *et al.*, 2005; Seelaar *et al.*, 2008; Hodges *et al.*, 2010). Therefore, further clinical studies of patients with the repeat expansion are needed to confirm our observation and to elucidate the genetic contribution in semantic dementia. In contrast to a study by Lillo *et al.* (2010) that reported that psychotic symptoms are a common feature in FTD + ALS, hallucinations were reported in only two patients with the repeat expansion in the present series and delusions in none.

Predominant temporal atrophy on neuroimaging is a frequent (40.6%) finding in the present series of patients carrying the repeat expansion, especially in those with PPA. This clearly contrasts with the frontal or frontotemporal pattern of atrophy in chromosome 9p-linked families, and with the absence of a specific atrophy pattern for FTD + ALS in a correlative voxel-based morphometry study (Le Ber *et al.*, 2009; Rohrer *et al.*, 2010; Boxer *et al.*, 2011; Pearson *et al.*, 2011). However, Coon *et al.* (2011) also found a trend towards more temporal atrophy in patients with language-dominant FTD + ALS than in those with behavioural-dominant FTD + ALS, and severe and circumscribed atrophy of the anterior temporal lobes has also been described in a case report of FTD + ALS (Kuwahara *et al.*, 2010). Therefore, a voxel-based morphometry study in a large series of patients carrying the pathogenic repeat expansion is required to confirm our observation of temporal involvement.

The neuropathological findings were consistent with type B FTLD-TDP pathology, with characteristic ubiquitin- and p62-pathology in the granular layer of the cerebellar cortex, in all except for two brains. This cerebellar pathology has been found in other families with FTD + ALS with the pathogenic repeat expansion (Polvikoski *et al.*, 2003; Boxer *et al.*, 2011; DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011), and in a series of patients with FTD + ALS or FTLD-TDP (King *et al.*, 2011). Our observation confirms its strong association with the pathogenic repeat expansion, but it is not an absolute requisite for this disorder. Whether the cerebellar pathology is pathognomic for the repeat expansion has to be investigated in future studies. The TDP-43-negative staining probably indicates that the involvement of the TDP-43 protein is more downstream in the formation of these inclusions, whereas the p62 protein as a non-specific protein reflects the degradation of ubiquitinated proteins via the ubiquitin proteasome system (King *et al.*, 2011).

In contrast to ubiquitin staining of neuronal inclusions, TDP-43- and p62-positive glial inclusions in the subcortical white matter found in several of the present brains did not stain with ubiquitin antibody, which is in accordance with the observations in other studies (Hiji *et al.*, 2008; Zhang *et al.*, 2008). TDP-43- and

p62-positive astrocytic inclusions in a single brain from our youngest patient with FTD + ALS were ubiquitin negative as well, as also mentioned by Zhang *et al.* (2008). Perhaps, these lesions merely reflect that glial abnormalities are associated with a faster progression of the same disease process, instead of a different underlying pathophysiology. Giebus *et al.* (2010) reported asymmetric TDP pathology in a PPA case, we could not confirm this in our patient with PPA, as only the right hemisphere was available for immunohistochemistry.

Neuronal intranuclear inclusions were found in several of our patients with the pathogenic repeat expansion, as has previously been found in familial FTD + ALS (Bigio *et al.*, 2004; Seelaar *et al.*, 2007). The prediction of Bigio *et al.* (2004) that these inclusions might be associated with a repeat expansion disorder has been confirmed. Using a different method, we could not confirm the results from DeJesus-Hernandez *et al.* (2011) who demonstrated the specific presence of RNA-positive foci in the nucleus of frontal cortex neurons using a (GGCCCC)<sub>4</sub> Cy3-labelled oligonucleotide probe. Nevertheless, the nuclear localization of the disease process was already suggested by the presence of TDP-43-positive intranuclear inclusions in a few of the present brains, and the absence of cytoplasmic abnormalities of C9orf72 protein in brain carrying the pathogenic repeat expansions. However, it is known from other non-coding expanded repeat disorders that repeat expansions in the transcripts may result in cellular toxicity and the formation of RNA foci of distinct morphology and size (Wojciechowska and Krzyzosiak, 2011). The mutant messenger RNA may interact with specific RNA-binding proteins, and sequestration of RNA-splicing factors and RNA binding proteins may lead to disruption of nuclear processes, including transcription, splicing or messenger RNA processing (Todd and Paulson, 2010). Another possible pathogenic mechanism underlying disease is loss-of-function, which is supported by reduced expression in one of the three transcripts of C9orf72, as demonstrated in the study of DeJesus-Hernandez *et al.* (2011). However, their findings have to be confirmed in future studies. Further studies to clarify these mechanisms may hopefully provide pharmacological target for preventing or delaying the disease.

A few limitations of this study have to be addressed. First of all, our observations on the frequency and phenotype of the repeat expansion are confined to FTD, as patients with ALS were not included in this study. Secondly, the finding of predominant temporal atrophy in a subset of patients carrying the repeat expansion was semi-quantitatively assessed, and should be confirmed by voxel-based morphometry in another cohort.

In conclusion, the hexanucleotide repeat expansion in C9orf72 is an important genetic cause of FTD and FTD + ALS. It will be a challenge to explain the wide variation in clinical phenotype of this genetic defect, including behavioural variant FTD, ALS and PPA. In addition, it would be interesting to determine whether the severe glial involvement upon the uniform TDP-43 pathology found in some cases has a pathophysiological significance or is just an epiphenomenon. Hopefully, revealing these underlying mechanisms of the repeat expansions will lead to the development of therapeutic interventions for this devastating disease.

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## Supplementary material

Supplementary material is available at *Brain* online.

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