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Pattern of ubiquilin pathology in ALS and FTLD indicates presence of *C9ORF72* hexanucleotide expansion

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Abstract

C9OF72-hexanucleotide repeat expansions and ubiquilin-2 (*UBQLN2*) mutations are recently identified genetic markers in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). We investigate the relationship between *C9ORF72* expansions and the clinical phenotype and neuropathology of ALS and FTLD. Genetic analysis and immunohistochemistry (1HC) were performed on autopsy-confirmed ALS ($N = 75$), FTLD-TDP ($N = 30$), AD ($N = 14$), and controls ($N = 11$). IHC for neurodegenerative disease pathology consisted of C9ORF72, UBQLN, p62, and TDP-43. A *C9ORF72* expansion was identified in 19.4 % of ALS and 31 % of FTLD-TDP cases. ALS cases with *C9ORF72* expansions frequently showed a bulbar onset of disease (57 %) and more rapid disease progression to death compared to non-expansion cases. Staining with *C9ORF72* antibodies did not yield specific pathology. UBQLN pathology showed a highly distinct pattern in ALS and FTLD-TDP cases with the *C9ORF72* expansion, with UBQLN-positive cytoplasmic inclusions in the cerebellar granular layer and extensive UBQLN-positive aggregates and dystrophic neurites in the hippocampal molecular layer and CA regions. These UBQLN pathologies were sufficiently unique to allow correct prediction of cases that were later confirmed to have *C9ORF72* expansions by genetic analysis. UBQLN pathology partially co-localized with p62, and to a minor extent with TDP-43 positive dystrophic neurites and spinal cord skein-like inclusions. Our data indicate a pathophysiological link between *C9ORF72* expansions and UBQLN proteins in ALS and FTLD-TDP that is associated with a highly characteristic pattern of UBQLN pathology. Our study

indicates that this pathology is associated with alterations in clinical phenotype, and suggests that the presence of *C9ORF72* repeat expansions may indicate a worse prognosis in ALS.

Keywords

Amyotrophic lateral sclerosis; Frontotemporal lobar degeneration; C9ORF72; UBQLN2; UBQLN1

Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are fatal neurodegenerative diseases without effective treatments. ALS is the most frequent adult onset motor neuron disease characterized by rapidly progressive paresis leading to death with a mean survival of ~ 3 years following onset of symptoms [21]. FTLD is the second most frequent cause of dementia and in people <65 years old, its prevalence is similar to Alzheimer's disease (AD) [44]. TDP-43 is the major component in ubiquitin-positive neuronal inclusions in sporadic ALS (sALS) and the largest subset of FTLD now known as FTLD-TDP [14, 39, 40]. Indeed, ALS and FTLD-TDP are thought to represent a continuum of different manifestations of the same underlying disease, i.e. TDP-43 proteinopathy. ALS is mostly sporadic but ~ 10 % of cases have a first- or second-degree relative with the disease suggestive of familial inheritance of ALS (fALS). Over ten genes have been found to harbor mutations that cause fALS, with mutations in *SOD1*, encoding the Cu/Zn superoxide dismutase, *TARDBP*, fused in sarcoma *(FUS)* and the optineurin *(OPTN)* gene accounting for ~ 30 % of these cases [9, 19, 28, 43, 47, 51, 57]. Mutations in *TARDBP* and *FUS* are occasionally observed in FTLD cases as well [27, 56], Moreover, mutations in the ubiq-uilin-2 (*UBQLN2*) gene encoding the ubiquitin-like protein UBQLN2 were shown to cause very rare cases of domi-nantly inherited, chromosome-X-linked ALS and ALS with dementia [8]. UBQLN is a member of the ubiquitin-like protein family (ubiquilins) that participates in degradation of proteins through the proteasome and autophagy pathways [24, 31, 37, 46]. Humans contain four ubiquilin genes, each encoding a separate protein. The expression pattern of these genes in humans is not known, although studies suggest that UBQLN1 has a more widespread expression than UBQLN2, and that UBQLN3 is only expressed in the testis [6, 34, 63].

Importantly, two recent studies identified a non-coding GGGGCC hexanucleotide repeat expansion in the *C9ORF72* gene as the most common genetic abnormality in familial and sporadic ALS/FTLD [7, 42]. The repeat expansion was found to be associated with a selective reduced expression of one of the *C9ORF72* transcripts [16]. It was suggested that aberrant promoter function, aberrant splicing of *C9ORF72* primary transcripts or sequestration of RNA-binding proteins could be possible pathogenic consequences of *C9ORF72* mutations [7, 16].

Despite these important findings on identifying disease genes associated with ALS/FTLD, few studies have systematically analyzed the pathology of ALS/FTLD with *C9ORF72* repeat expansions [1, 3, 16, 36, 49, 50, 52, 54] or with *UBQLN2* mutations [8]. Previous studies have shown that ALS/FTLD cases with *C9ORF72* expansion cases do not contain protein aggregates that comprise C9ORF72 protein [7, 42], although TDP-43 inclusions are present and p62 was hypothesized to be the major disease pathology since p62-immunoreactive neuronal cytoplasmic inclusions in the cerebral cortex, basal ganglia, hippocampus, and cerebellum were detected in cases with *C9ORF72* repeat expansions [1, 54]. Here, we describe neuropathological findings in a large and clinically well-defined

cohort of ALS and FTLD-TDP autopsy cases and controls, and evaluate the relevance of *C9ORF72* and *UBQLN2* gene mutations to the neuropathological and clinical phenotypes.

Methods

Autopsy cohort

Individuals who underwent autopsy in the Center for Neurodegenerative Disease Research at the University of Pennsylvania from 2001 to 2010 were included. Our cohort was composed of 75 patients with a clinical diagnosis of ALS in accordance with the modified El Escorial Criteria [4] and a confirmed neuropathological diagnosis of ALS (Table 1). Detailed clinical characteristics [age at onset, age at death, site of onset, disease duration, ALS global disease severity as measured by a functional rating score (ALSFRS-R) [5], the Medical Research Council sumscore (MRCS) [25], gender, performance on cognitive tests] were ascertained by retrospective chart review of clinical visits within the University of Pennsylvania Health System; the vast majority of patients were seen by two neurologists (L.E., L.M.). Unless otherwise specified, results of clinical testing used in this study were from the visit most proximate to death, occurring within 3 months of death. Of the ALS cases included here, ten had a clinical history of dementia (ALS-D) and met criteria for FTLD [17, 38, 41]. Thirteen of the ALS cases had a family history of ALS (fALS). We furthermore included 30 cases with a neuropathological diagnosis of FTLD-TDP [33], 14 cases with a neuropathological diagnosis of AD [18], and 11 normal controls (CTRL) age-matched to the ALS group.

Basic neuropathological characterization

Pathology was examined in the following regions of the central nervous system (CNS): amygdala, hippocampus (dentate gyrus, molecular layer, and CA regions/subiculum), middle frontal gyrus, superior or middle temporal gyms (SMT), motor cortex, cerebellum, cervical spinal cord (CSC), and lumbar spinal cord (LSC, tissue for this region was available for ALS cases only).

Sections were fixed and cut into 6–7 μm sections, stained with hematoxylin and eosin (HE) and Thioflaviu S, and immunohistochemistry (IHC) was performed with antibodies to tau, Aβ, α-synuclein, ubiquitin, and TDP-43 as previously described [13, 14, 39, 40], The extent of TDP-43, tau, Aβ pathology, and neuronal loss (as monitored by HE) was rated for each region on a four-point ordinal scale (0, none; 1, mild; 2, moderate; 3, severe/numerous) as previously described [15, 55].

Characterization of UBQLN and C9ORF72 pathology

IHC for UBQLN was performed using two antibodies to UBQLN2: a commercially available mouse monoclonal antibody (MAb) (clone 5F5, H00029978-M03, Abnova, Walnut, CA, USA) used at 1:20,000 dilution, and a polyclonal rabbit antibody (AP12092PU-N, Acris Antibodies, San Diego, CA, USA), used at 1:100 dilution [8], The two antibodies were generated against C- and N-terminal portions of UBQLN2, respectively, but are likely to crossreact with all four UBQLN proteins because the regions used to generate the antibodies are highly conserved in all UBQLN proteins. In addition to these antibodies we used a polyclonal rabbit anti-UBQLN1-specific antibody raised by our lab to a synthetic peptide corresponding to amino acids 501–510 in human UBQLN1, a sequence that is not present in UBQLN-2, UBQLN-3 or UBQLN-4 proteins, used at 1:10,000 dilution. IHC for C9ORF72 pathology was performed using a commercially available rabbit polyclonal anti-*C9ORF72* ab (HPA023873, Sigma-Aldrich, St. Louis, MO, USA), 1:1,000, similar to the procedure as described above [7], For p62 IHC, a

commercially available mouse MAb (Abnova, Walnut, CA, USA) was used at 1:500 and with microwave antigen retrieval [1, 23].

Double-labeling immunofluorescence (IF) analyses were performed as previously described [40] using Alexa Fluor 488- and 594-conjugated secondary ab (Molecular Probes, Eugene, OR, USA), treated for autofluorescence with Sudan Black solution [48], and coverslipped with Vecta-shield-DAPI mounting medium (Vector Laboratories). Fluorescence images were obtained using a Leica TCS SPE-II scanning laser confocal microscope.

Neuropsychological testing

Ante-mortem cognitive testing was performed at 3- to 6-month intervals during routine clinic visits. Data for three cognitive tests were available for a subset of the autopsy cohort (n = 55/75 ALS cases, 17/30 FTLD-TDP cases) within 12 months of death. These included a test of letter-guided verbal fluency (F-words test) [29], a test of frontal executive function (Oral Trail-Making Test) [30] assessing information processing speed and the capacity to maintain a complex mental set, and the Mini-Mental State Examination (MMSE) [12]. For the letter fluency test, patients without significant dysarthria were asked to generate as many unique words (proper nouns and numbers excluded) beginning with the letter “F” in 1 min. Patients with dysarthria, but showing preserved limb and hand function, were asked to write the words; 90°s was allotted to these patients. The total number of unique words produced was recorded. For the Oral Trail-Making Test, patients without significant dysarthria were asked to sequentially say letters of the alphabet, beginning with A, alternating with numbers, beginning with 1, in an ascending order (i.e. A–1, B–2) and ending at Z–26. Each error was subtracted from the maximum score of 52. Patients with significant dysarthria, but with relatively preserved hand and limb ability, were asked to perform the same task in writing. We also included the Frontal Behavioral Inventory (FBI), a 24-item caregiver-based behavioral questionnaire designed for the diagnosis and quantification of FTD symptoms [20]. Data for neuropsychological tests were unavailable in the subgroup of FTLD with *C9ORF72* expansion, therefore no analysis of FTLD-TDP subgroups with regard to neuropsychological data is provided here.

Genetics methods

Genomic DNA was extracted from peripheral blood or brain tissue following the manufacturer's protocols [Flex-igene (Qiagen, Valencia, CA, USA) or QuickGene DNA whole blood kit L (Autogen, Holliston, MA, USA) for blood, and QIAsymphony DNA Mini Kit (Qiagen) for brain]. Genotyping for a C9ORF72 repeat expansion was performed as described by Renton et al. [42] with some modifications. Briefly, repeat-primed PCR was performed using 100 ng of DNA in a final volume of 28 μl containing (final concentrations): Roche (Indianapolis, IN, USA) FastStart PCR Master Mix (1×), DMSO (7 %, Sigma-Aldrich, St. Louis, MO, USA), betaine (0.93 M, Sigma-Aldrich, St. Louis, MO, USA), deazaGTP (0.18 mM, Roche, Indianapolis, IN, USA), $MgCl_2$ (0.9 mM, Sigma-Aldrich, St. Louis, MO, USA), and 10× primer mix (1×). The 10× primer mix was prepared containing 14 μM 6-FAM-labeled forward primer (6-FAM-5′ AGTCGCTAG AGGCGAAAGC), 7 μM reverse repeat primer (5′TACGC ATCCCAGTTTGAGACGGGGGCCGGGGCCGGGGCC GGGG), and 14 μM anchor tail reverse primer (5′TACG CATCCCAGTTTGAGACG). Touchdown PCR cycling conditions consisted of 4 min at 95 °C followed by cycles of 95 °C for 30 s, annealing between 70 and 56 °C for 1 min, and extension at 72 °C for 3 min, ending with a final extension step of 10 min at 72 °C. The annealing temperature is decreased by 2 °C in each step starting at 70 °C for two cycles, 68 °C for three cycles, 66 °C for four cycles, 64 °C for five cycles, 62 °C for six cycles, 60 °C for seven cycles, 58 °C for eight cycles, and 56 °C for five cycles. PCR product (2 μl) was mixed with 0.5 μl of ROX 500 Size Standard (Life Technologies,

Carlsbad, CA, USA) and 7.5 μl Hi-Di formamide (Life Technologies, Carlsbad, CA, USA) and evaluated on an ABI 3130 capillary electrophoresis instrument with POP7 polymer and a 36 cm capillary with a 23 s injection time. Interpretation of a positive expansion case was based on the presence of a stutter pattern while that of a case lacking the expansion produced one or more peaks with an abrupt ending peak. In some cases the absence of an expansion was confirmed using a standard two primer PCR reaction across the repeat region, The presence of two unique peaks was interpreted as negative for a repeat expansion while the identification of only a single peak was not informative. This two primer genotyping was performed using primers from by Dejesus-Hernandez et al. [7] with minor protocol modifications. Briefly, the PCR was performed using 50 ng of DNA in a final volume of 20 μl containing (final concentrations): Amplitaq Gold buffer (1×), DMSO (5 %), betaine (1 M), dNTP mixture with 7-deazaGTP instead of dGTP (0.25 mM each), $MgCl_2$(0.9 mM), forward and reverse primers (1 μM each), and Amplitaq Gold polymerase 0.5 U/reaction (Life Technologies, Carlsbad, CA, USA). PCR cycling conditions consisted of 10 min at 94 °C followed by 36 cycles of 94 μC for 35 s, annealing between 62 μC for 2 min, and extension at 72 °C for 1 min, ending with a final extension step of 10 min at 72 °C. The ABI 3130 electrophoresis conditions for this assay are the same as for the repeat-primed PCR reaction. The coding region of *UBQLN2* was sequenced as described by Deng et al. [8] in ALS and FTLD-TDP cases with DNA available. Sequence data were analyzed using Mutation Surveyor software (SoftGenetics, State College, PA, USA).

Statistical analysis

Data analysis was performed using SPSS (Version 17.0 SPSS Inc., Chicago, IL, USA). The "average" (and "range") of data on patient characteristics was estimated by calculating the median (and 25th–75th percentiles). Differences between two groups were compared using Wilcoxon Mann–Whitney test. To compare raw data of multiple groups, Kruskal–Wallis analysis of variance on ranks was applied, followed in case of significance by Dunn's Method. Trend analysis was conducted using the Mantel–Haenszel Chi-square test. All correlations were studied using Spearman's rank order correlation coefficient. Bonferroni-correction for multiple testing was applied when contrasts were not driven by a specific hypothesis. For all other tests, P values <0.05 were considered significant. All statistical tests were two-sided.

Results

Genetic analysis of *C9ORF72* and *UBQLN2*

To identify FTLD and ALS cases with either a *C9ORF72* hexanucleotide repeat expansion or *USQLN2* gene mutation in our cohort, we analyzed all sporadic and familial autopsy cases with FTLD-TDP (n = 29) and ALS (n = 72) for which a DNA sample was available (for 1 FTLD-TDP case and 3 ALS cases, no DNA sample was available).

A *C9ORF72* hexanucleotide repeat expansion was identified in 19,4 % (14/72) of ALS cases, and in 31.0 % (9/29) of FTLD-TDP cases. For the subset of autopsy cases in which information about family history was known, a *C9ORF72* expansion was identified in 42.9 % (6/14) of ALS cases with a family history of ALS and/or FTLD in first- or second- degree relatives. The *C9ORF72* expansion rate was 23.1 % (3/13) in ALS cases with a family history of neurodegenerative diseases other than ALS or FTLD suggesting that the phenotype for *C9ORF72* expansions may be broad. *C9ORF72* expansions were also identified in 11.6 % (5/43) apparent sporadic ALS cases; however, this number may be high due to lack of complete family history. Similarly, a *C9ORF72* expansion was identified in 33.3 % (6/18) of FTLD-TDP cases with a family history of any neurodegenerative disease, although the number of total cases with information is too small to draw conclusions or

make comparisons. No expansions were identified in a small number of apparent sporadic FTLD-TDP cases ($n = 6$). The ethnic composition of the cohort was approximately 26 % white Latino, 67 % Caucasian, and 2 % Black, and 5 % other or unknown. Of those with known ethnicity or race ($n = 99$), *C9ORF72* expansions were identified in 8.1 % white Latinos (8/99) and 15.1 % Caucasian non-Latinos (15/99). The cohort included five cases with progranulin (*GRN*) mutations and two with *SOD1* mutations, none of which were found to have a *C9ORF72* expansion.

Since UBQLN2 mutations have been associated with ALS, the *UBQLN2* gene was sequenced in all ALS and FTLD-TDP cases ($n = 101$) for which DNA was available, to rule out mutations as a cause of disease. No pathogenic *UBQLN2* mutations were identified in the cases sequenced; however, a variant of uncertain significance (c.998G>T, p.Ala330Ser) was identified in a FTLD-TDP case. An affected family member was tested and did not carry the variant, therefore it is not likely to be the cause of FTLD in the family.

Clinical phenotype in ALS and FTLD-TDP with/without *C9ORF72* expansion

Having identified several ($n = 14$) ALS and ($n = 9$) FTLD-TDP autopsy cases with a *C9ORF72* repeat expansion within our cohort, we determined if the presence of this repeat expansion was linked to the clinical phenotype. We observed the presence of the *C9ORF72* expansion to correlate with several clinical variables, especially in the subgroup of cases with ALS. Data on disease duration were available for 66 ALS autopsy cases (it was missing for 1 *C9ORF72* expansion case and for 8 non-expansion cases). Disease duration to death was significantly shorter in ALS with *C9ORF72* expansion as compared to non-expansion cases ($P = 0.016$; Fig. 1). In contrast, no significant difference regarding disease duration, age of onset or age at death was observed in FTLD-TDP with/without *C9ORF72* expansion ($P > 0.05$).

A bulbar onset of disease was significantly more frequent in the *C9ORF72* expansion ALS cases as compared to non-expansion cases ($P = 0.04$, data on site of onset was missing for 1 non-expansion case). Of 14 ALS cases with *C9ORF72* expansion, 8 (57.1 %) showed a bulbar onset of disease, as compared to 21 % in the subgroup of non-expansion cases. Four of the eight ALS *C9ORF72* expansion cases with a bulbar onset showed bulbar LMN clinical signs, two presented with a bulbar UMN clinical picture and for two patients the data were incomplete. No significant difference with regard to gender or clinical rating scales (ALSFRS-R, MRCS) was observed between ALS or FTLD-TDP cases with/without *C9ORF72* expansion. Furthermore, no difference with regard to neuropsychological tests of executive function (F-words, Oral Trail-making test) or memory function (MMSE) could be detected between ALS with/without *C9ORF72* expansion ($P > 0.05$ each).

Description of C9ORF72 pathology

As the presence of *C9ORF72* repeat expansions in ALS was associated with distinct alterations in clinical phenotype, we examined if a neuropathological disease signature exists that could distinguish ALS and FTLD-TDP cases with and without *C9ORF72* expansion. We first analyzed C9ORF72 immunoreactivity in IHC, using a commercially available rabbit polyclonal anti-C9ORF72 ab which detects both the long (481 amino acid protein, isoform a) and the shorter isoform (222 amino acid protein, isoform b) of proteins encoded by *C9ORF72* [7] (Supplementary figure 1). Similar to Dejesus et al. [7], we observed a coarse punctate staining of synaptic terminals mainly in the CA4 region of the hippocampus in ALS, FTLD-TDP, AD, and CTRL without any difference in extent or regional distribution between the groups (Supplementary figure 2).

UBQLN pathology in subgroups of ALS and FTLD-TDP with/without *C9ORF72* repeat expansion

As C9ORF72 staining itself did not reveal any compelling differences between ALS and FTLD-TDP cases with or without *C9ORF72* expansion, we examined other types of pathology including UBQLN, TDP-43, and p62 through IHC in the entire cohort of autopsy cases.

Intriguingly, we observed UBQLN pathology in ALS and FTLD-TDP cases with a *C9ORF72* expansion to be highly distinct and to allow the differentiation of expansion cases from non-expansion cases with the pathologist blinded to the genetics results and based on the analysis of UBQLN pathology only. In the hippocampus of FTLD-TDP and ALS with *C9ORF72* expansion, dystrophic neurites that showed focal swellings and dot-like stipples (Fig. 2) and irregular, aggregate-like formations were extensive in the hippocampal molecular layer and in the CA1–CA4 region. In contrast, non-expansion cases hardly showed any hippocampal molecular layer pathology; instead, UBQLN pathology in non-expansion cases was restricted to the CA regions and the entorhinal cortex. Furthermore, dentate gyrus cytoplasmic neuronat inclusions (Fig. 2) were much more frequent in ALS and FTLD-TDP with *C9ORF72* expansion as compared to non-expansion cases (Fig. 3). These inclusions were observed in both ALS-D ($n = 10$) as well as in ALS without dementia ($n = 65$), though they were not observed in any AD or CTRL case.

In the cerebellum, all ALS and FTLD-TDP *C9ORF72* expansion cases showed round to crescent-shaped cytoplasmic neuronal inclusions in the granular layer (Fig. 3). In contrast, such inclusions were not observed in any of the ALS or FTLD-TDP non-expansion cases or in any control case. No further inclusions in the Purkinje cells or other cerebellar layers were observed, neither was there any relevant cerebellar neuronal loss.

In neocortical areas, FTLD-TDP and ALS with *C9ORF72* expansions showed dystrophic neurites and (to a lesser extent) aggregate-iike formations throughout the neocortex, while in non-expansion cases pathology was largely limited to superficial neocortical layers (e.g. cortical layers 2 and 3). In addition, neocortical pathology (as shown in Fig, 2) was much more extensive in the *C9ORF72* expansion cases. Neocortical dystrophic neurites were also found in AD cases located close to amyloid plaques. Spinal cord anterior horn skein-like to solid cytoplasmic inclusions were observed in both ALS with and without *C9ORF72* expansion and did not contribute to the differentiation of expansion versus non-expansion cases. They were not observed in FTLD-TDP or normal controls.

As UBQLN pathology showed a characteristic signature in ALS and FTLD-TDP with *C9ORF72* repeat expansion, we quantified UBQLN pathology in expansion and non-expansion cases using a semi-quantitative rating scale. UBQLN pathology was significantly more extensive in ALS and FTLD-TDP cases with *C9ORF72* expansion as compared to non-expansion cases in all the regions analyzed here ($P < 0.05$ for each area) with the exception of the spinal cord (Fig. 4). Cases with a *GRN* or *SOD1* mutation did not show differences in UBQLN pathology to cases with ALS or FTLD without C9ORF72 expansion.

We also analyzed a possible difference of other types of pathology in subgroups of ALS and FTLD-TDP with and without *C9ORF72* repeat expansion. The extent of ubiquitin pathology was significantly more extensive in ALS and FTLD-TDP cases with a *C9ORF72* expansion as compared to non-expansion cases in the middle frontal gyrus and SMT ($P < 0.05$ each). No significant difference of tau, Aβ, or α-synuclein pathology was observed between ALS and FTLD-TDP with and without a *C9ORF72* expansion.

Characterization of UBQLN pathology by UBQLN subtypes, TDP-43 and p62

To further characterize the composition of the distinct pathology in ALS and FTLD-TDP with *C9ORF72* repeat expansion, we performed IHC and double-labeling IF for UBQLN subtypes, TDP-43 and p62.

The UBQLN2 antibodies used here were likely to cross-react with other UBQLN proteins because the regions used to generate the antibodies are highly conserved in all four UBQLN subtypes. We observed the UBQLN2 antibodies to detect both UBQLN2 and UBQLN1 (Supplementary figure 3), whereas the polyclonal rabbit anti-UBQLN1-specific antibody did not cross-react with UBQLN2 (antibodies specific for the other UBQLN proteins are not available). Immunostaining with the UBQLN1-specific antibody exhibited considerable overlap with immunostaining using the antibody that does not discriminate between the different UBQLN isotypes, especially in irregular, aggregate-like formations (e.g. in the hippocampus; Fig. 5) and focal swellings of dystrophic neurites. However, the UBQLN1 antibody did not appear to stain the neuronal cytoplasmic inclusions in certain regions of the brain that were easily seen with the Pan-UBQLN antibody (e.g. in the hippocampal dentate gyrus; Fig. 5). It is possible that these inclusions do not contain UBQLN1 or that the UBQLN1 epitope is masked.

We next analyzed the relation of UBQLN pathology to TDP-43, for which distinct subtypes regarding the distribution of pathology in the cortices of FTLD-TDP have been described [32]. While three different types of TDP-43 pathology were observed in FTLD cases without the expansion (7 type A, 9 type B, and 5 type C), curiously, FTLD-TDP with *C9ORF72* expansion mainly (8/9) showed type B. In ALS and FTLD-TDP cases with *C9ORF72* expansion, UBQLN pathology was much more extensive than TDP-43 pathology in the regions examined here. Double-labeling IF analyzed by confocal microscopy showed that hippocampal dentate gyrus inclusions and molecular layer aggregate-like formations immunoreactive for UBQLN did not co-stain with TDP-43 antibodies (Fig. 5). In focal swellings of dystrophic neurites in neocortical areas of ALS and FTLD-TDP, UBQLN and TDP-43 pathology were observed to be located close to one another, though they rarely overlapped. When they did, high-resolution confocal microscopy demonstrated that in several of these lesions, a core of UBQLN-positive structures seemed to be surrounded by TDP-43 immunoreactive material (Fig. 5).

As p62 pathology has previously been reported [1, 52, 54] in ALS and FTLD with *C9ORF72* expansion, we analyzed its relation to UBQLN pathology. In both the hippocampus and cerebellum of ALS and FTLD-TDP cases with *C9ORF72* expansion, several inclusions exhibited colocalization of UBQLN and p62 pathology in both hippocampal aggregate-like formations as well as in cerebellar granular layer neuronal inclusions (Fig. 5). However, UBQLN pathology was more extensive than p62 pathology throughout the areas analyzed here (only a minor part of the pathology immunoreactive for UBQLN also showed immunoreactivity to p62, as is shown for cerebellar granular layer inclusions and hippocampal molecular layer aggregate-like formations in Fig. 5).

Relation of UBQLN pathology to other pathology and clinical phenotype

As we observed distinct UBQLN pathology in ALS and FTLD-TDP with *C9ORF72* expansion, we analyzed its relation to other pathology as well as to clinical parameters in the entire cohort of ALS and FTLD-TDP.

The extent of UBQLN pathology as determined on a semi-quantitative rating scale correlated with neuronal loss in the hippocampus ($R = 0.37$, $P = 0.03$) and in the motor cortex ($r = 0.59$, $P = 0.028$) of ALS. The extent of ubiquitin pathology increased with the extent of UBQLN pathology in the amygdala ($R = 0.6$, $P = 0.022$) and primary motor cortex

($R = 0.82$, $P = 0.014$) of ALS. We also analyzed a possible relation of UBQLN pathology to the clinical phenotype in the entire cohort of ALS and FTLD-TDP. In ALS, more extensive SMT UBQLN pathology correlated with a shorter duration of disease ($R = -0.41$, $P = 0.004$), though this relation failed to reach significance in the motor cortex ($P = 0.05$) or in any other region. In contrast, no correlation of UBQLN pathology with age at onset or age at death could be observed ($R < 0.4$, $P > 0.5$ each). No direct correlation of UBQLN pathology with tests of executive function (F-words test, Oral Trail-making test) or memory (MMSE) was observed in ALS or FTLD-TDP ($R < 0.4$, $P > 0.05$ each). The extent of UBQLN pathology showed no correlation with clinical rating scales (MRCS, ALSFRS-R) in ALS ($R < 0.4$, $P > 0.05$ each).

Discussion

This study confirms *C9ORF72* repeat expansions to be frequent in ALS and FTLD-TDP. It furthermore shows the presence of *C9ORF72* expansions in ALS to be linked to alterations in clinical phenotype, including a shorter duration of disease. Importantly, this study demonstrates that while staining for C9ORF72 does not identify expansion cases, UBQLN pathology provides a characteristic neuropathological disease signature that distinguishes ALS and FTLD-TDP cases with and without *C9ORF72* expansion, suggesting a pathophysiological link between *C9ORF72* and UBQLN pathology.

As we expected based on previous studies, the frequency of identified *C9ORF72* repeat expansions in autopsy confirmed ALS and FTLD-TDP was relatively high, particularly in familial cases. The frequency of 42.9 % in fALS was comparable to that seen by Gijselinck et al. [16] (47 %). For FTLD there is a wider variation of expansion frequency in the literature by cohort (22.5–61.5 % in FTLD-TDP [7] and 16 % in clinical FTLD [16], yet our finding of 31 % in FTLD-TDP in our relatively small cohort is similar.

We observed *C9ORF72* repeat expansion not only to be frequent in ALS and FTLD-TDP but also to be associated with alterations in clinical phenotype of ALS. Although previous studies have not linked *C9ORF72* expansions to a shorter survival in ALS [7, 16, 42], our study observed that ALS cases with *C9ORF72* expansions showed a significantly shorter disease duration as compared to non-expansion cases (Fig. 1), suggesting that the presence of *C9ORF72* expansions could be a negative prognostic indicator in ALS. However, the shorter disease duration of *C9ORF72* expansion ALS observed here could well have been influenced by the specific composition of our cohort as well as by missing data in nine of the ALS autopsy cases. Generally, a bulbar onset of disease is clinically associated with speech and swallowing difficulties early-on in the disease, and is usually observed in approximately 25 % of all ALS cases [21]. Remarkably, our subgroup of ALS with *C9ORF72* expansion showed a bulbar onset in over 50 % of cases, as compared to a significantly lower frequency in the subgroup of non-expansion cases (21 %). This high proportion of bulbar onset cases is in line with a previous study [52] and may contribute to the shorter disease duration of *C9ORF72* expansion cases observed here, since a bulbar onset is a well-known risk factor for a shorter survival in ALS [21]. As our finding of a shorter survival in ALS with a *C9ORF72* repeat expansion may have been influenced by the high proportion of bulbar onset cases in the expansion group and by some missing data, future studies will be necessary to confirm the relevance of the *C9ORF72* repeat expansion as a risk factor for a shorter survival in ALS.

To determine neuropathological correlates of the *C9ORF72* repeat expansion, we analyzed C9ORF72 pathology in ALS and FTLD-TDP. Using a commercially available antibody for IHC, we observed no difference in C9ORF72 immunoreactivity between cases with or without *C9ORF72* expansion (Supplementary figure 2). A previous study suggested the

transcription of *C9ORF72* leads to the expression of two different protein isoforms of C9ORF72 [7]. While a significant reduction of transcripts encoding the longer (481 amino acid) isoform was observed, no significant changes in total protein levels could be detected. As a possible explanation, the antibody used here was detecting both alternative isoforms of the protein C9ORF72 (Supplementary figure 1), and may therefore not be sensitive enough to reflect regional differences in protein composition of C9ORF72 affecting only a single isoform.

Having found that C9ORF72 pathology itself could not distinguish between ALS and FTLD-TDP cases with or without *C9ORF72* expansion, we examined other pathology including UBQLN. Though none of the ALS and FTLD-TDP cases in our cohort showed an *UBQLN* mutation, we observed UBQLN pathology to be widespread in ALS and FTLD-TDP (Fig. 3). This confirms findings of a previous study that UBQLN pathology occurs not only in fALS with *UBQLN2* mutations but also in sporadic cases [8]. One of the antibodies used here to identify UBQLN pathology was the same one that was used in the previous study and may cross-react with all four UBQLN proteins. Our results suggest that UBQLN1 might accumulate in a subset of the inclusions in which UBQLN proteins are found, or that its epitope might be masked under certain circumstances. It is also possible that different UBQLN proteins accumulate in the different inclusions. It will be important to determine if this is the case using antibodies specific for each of the isoforms, UBQLN proteins contain an N-terminal ubiquitin-like domain, which mediates interaction with the 19S subunit of the proteasome, and a C-terminal ubiquitin-associated domain, which binds poly-ubiquitinated proteins [26]. This structural organization is characteristic of proteins that deliver ubiquitinated proteins to the proteasome for degradation [11]. However, the proteins also function in autophagy, suggesting they play dual roles in disposing misfolded proteins through the proteasome and lysosomal pathways [45]. There is increasing evidence that different members of this family may play an important role in neurodegenerative diseases, including ALS [8], Huntington's disease [59] and AD [2, 22, 34, 35, 53, 58], Our study demonstrates that while some of the pathological alterations (e.g. dystrophic neurites, aggregate-like formations) seen in hippocampal and neo-cortical areas of ALS and FTLD-TDP contain material immunoreactive for UBQLN1, others (e.g. dentate gyrus cytoplasmic neuronal inclusions) are distinguishable by general UBQLN accumulation and/or pathology.

Importantly, and in contrast to staining with C9ORF72 antibodies, we observed ALS and FTLD-TDP cases with *C9ORF72* expansions to show a highly distinct pattern of UBQLN pathology that enabled the correct classification of cases with C9ORF72 repeat expansions by a pathologist blinded to the genetic analysis results. This finding suggests a pafhophysiological link between *C9ORF72* mutations and UBQLN pathology in ALS and FTLD-TDP. It is so far unclear how the *C9ORF72* hexamicleotide repeat expansion leads to pathology and how this may, in turn, affect other proteins like UBQLN. It is interesting to note that UBQLNs have been shown to bind proteins containing polyglutamine and polyalanine expansions [10, 60, 61], which might be related to the pathology seen with *C9ORF72* hexanucleotide repeat expansions. In normal individuals at least three alternatively spliced *C9ORF72* transcripts (variants 1–3) are expressed in the brain. Quantitative mRNA expression analysis indicated that the GGGGCC repeat expansion abolished *C9ORF72* transcript variant 1 expression, leading to an overall reduction in *C9ORF72* transcripts [7]. On a speculative level, changes caused by *C9ORF72* transcription could (through a yet unknown pathomechanism) be active further downstream in the pathological pathway of ALS and FTLD-TDP and affect the regional expression of proteins involved in handing over polyubiquitinated proteins to the shuttle factors for degradation. In line with this, previous studies analyzing the pathology of *C9ORF72* ALS and FTLD expansion cases observed inclusions immunoreactive of p62 (also called sequestosome 1), another protein that is believed to be involved in protein degradation in similar regions that

showed extensive UBQLN pathology here [1, 23, 49, 50, 54, 62]. Another possibility is that the expansions cause the encoded C9ORF72 protein to mis-fold, which might attract and dedicate UBQLN to its disposal thus diverting it UBQLN's vital functions. Proteins involved in shuttling proteins disposal like UBQLN and p62 could be reactively upregulated as a consequence of protein alterations further upstream in the pathological pathway activated by *C9ORF72* expansions. Further studies will be needed to elucidate the pathophysiological link between C9ORF72 and proteins involved in protein degradation.

On a speculative level, extensive UBQLN pathology in ALS *C9ORF72* expansion cases may also contribute to a shorter survival: supporting this hypothesis, we observed more extensive SMT UBQLN pathology to correlate with shorter disease duration in the entire ALS cohort. However, this relation just failed to reach significance in the primary motor cortex, and it could not be shown for other areas analyzed here, but this may reflect the limitations of a semi-quantitative rating system. Furthermore, we observed the extent of UBQLN pathology to correlate with neuronal loss in the primary motor cortex and hippocampus. Larger, multi-center studies linking genetics and pathology to survival will be necessary to clarify if the presence of *C9ORF72* expansions and/or UBQLN pathology is of prognostic relevance in ALS.

Taken together, our data suggest that *C9ORF72* gene mutations are pathophysiologically linked to UBQLN pathology in ALS and FTLD-TDP and that there are distinct patterns of UBQLN pathology in ALS and FTLD-TDP that predict the presence of *C9ORF72* repeat expansions. Our study furthermore indicates that this pattern of pathology is associated with alterations in clinical phenotype (including a frequent bulbar onset of disease), and suggests that the presence of a *C9ORF72* repeat expansion may indicate a worse prognosis in ALS.

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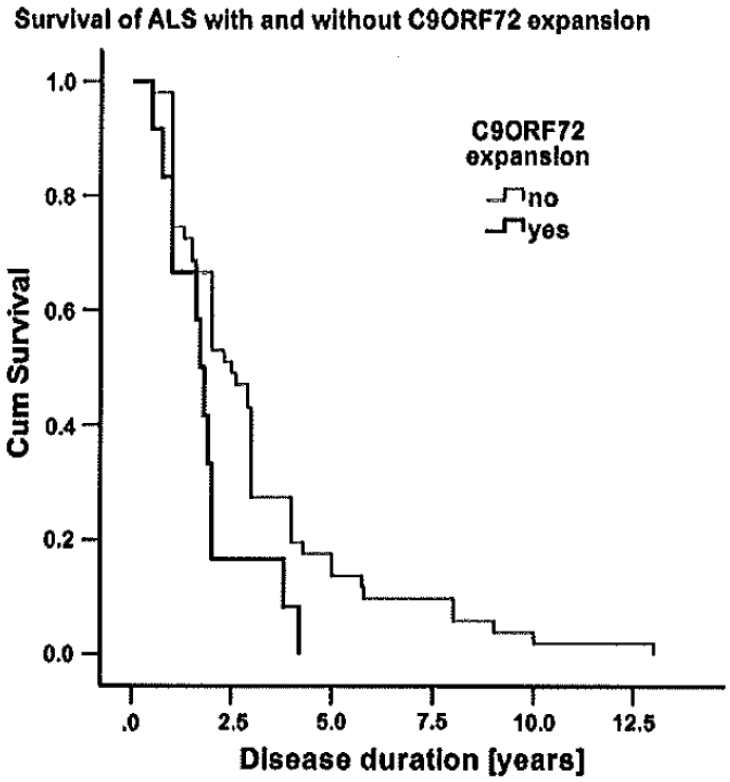

Fig. 1.

Survival in ALS with and without *C9ORF72* expansions. Kaplan-Meier plot shows survival of ALS autopsy cases with *C9ORF72* repeat expansion (*black curve, n* = 14) and without *C9ORF72* repeat expansion (*grey curve, n* = 61). Survival time was significantly shorter in cases with *C9ORF72* repeat expansion (*P* = 0.016)

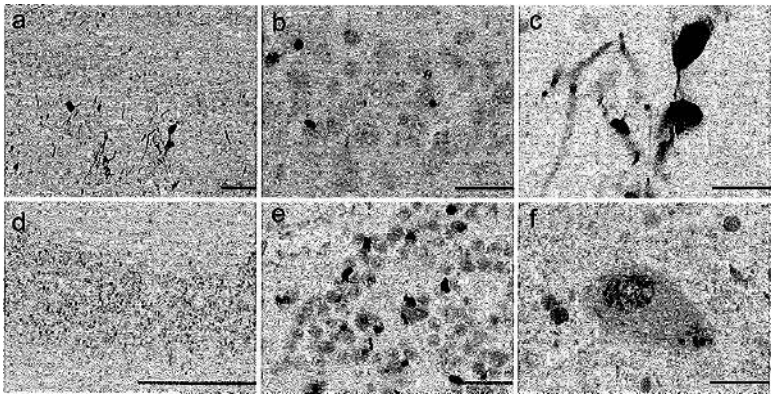

Fig. 2.

UBQLN pathology in ALS and FTLD-TDP. **a–c** Hippocampal dentate gyrus and molecular layer of an ALS case with *C9ORF72* expansion, **a** Dentate gyrus shows cytoplasmic inclusions that are depicted in **b** in higher resolution, dystrophic neurites with focal swellings as well as irregular, aggregate-like formations, which are depicted in **c** in higher resolution, **d** Cerebeliar molecular layer of an ALS case with a *C9ORF72* expansion showing cytoplasmic inclusions, which are shown in **e** in high resolution, **f** CSC anterior horn α-motoneuron of an ALS case witli a *C9ORF72* expansion showing skein-like to more solid cytopiasmic inclusions. *Scale bars* **a–c**, **e**, **f** 20 μm; **d** 200 μm

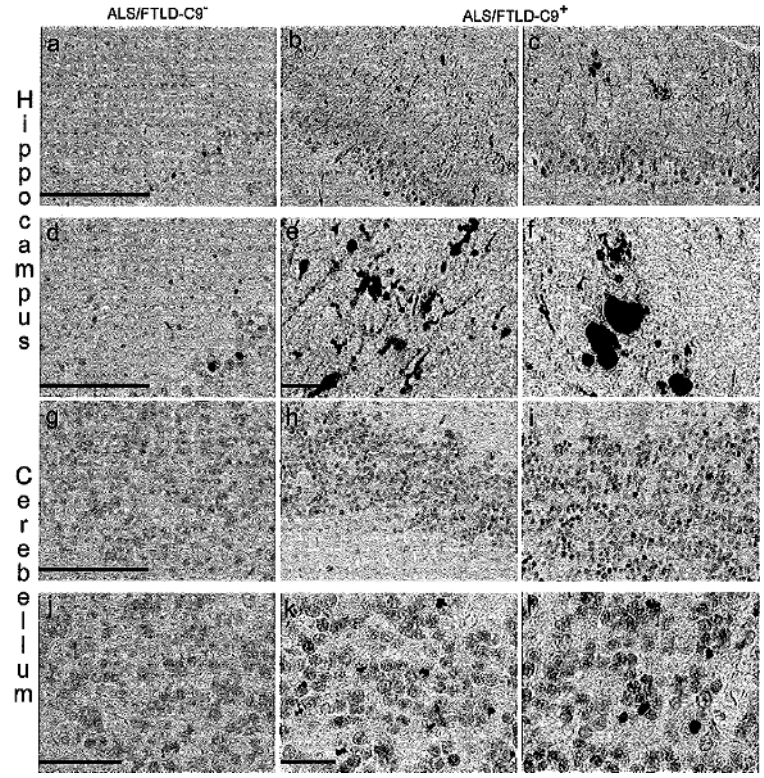

Fig. 3.

UBQLN pathology in subgroups of ALS and FTLD-TDP with/without C9ORF72 repeat expansion. The figure illustrates the differentiation of ALS and FTLD-TDP with *C9ORF72* expansion (ALS/FTLD-C9+) from non-expansion cases (ALS/FTLD-C9–) by UBQLN pathology. **a** Hippocampal dentate gyrus and molecular layer of a representative ALS case without *C9ORF72* repeat expansion. White dentate gyrus neuronal inclusions are detectable, the molecular layer is free of any pathology (**d** provides higher resolution image), **b**, **c** Extensive UBQLN pathology presenting with dystrophic neurites and aggregate-like formations in the molecular layer of two cases with *C9ORF72* repeat expansion (column from **b** downwards shows an ALS case, column from **c** downwards shows an FTLD-TDP case). **c**, **f** Higher resolution images of aggregate-like formations in the hippocampal molecular layer, **g**, **j** Cerebellar granular layer of a FTLD-TDP case without *C9ORF72* repeat expansion. The cerebellar granular layer does not show any pathology, **h**, **k** Cerebellar granular layer of an ALS case and an FTLD-TDP case (**i**, **l**) with *C9ORF72* repeat expansion shows cytoplasmic neuronal inclusions which were observed in all ALS/FTLD with *C9ORF72* repeat expansion. *Scale bars* **a–c**, **g–i** 200 μm; **d**, **j** 50 μm; **e**, **f**, *k*, 1 20 μm

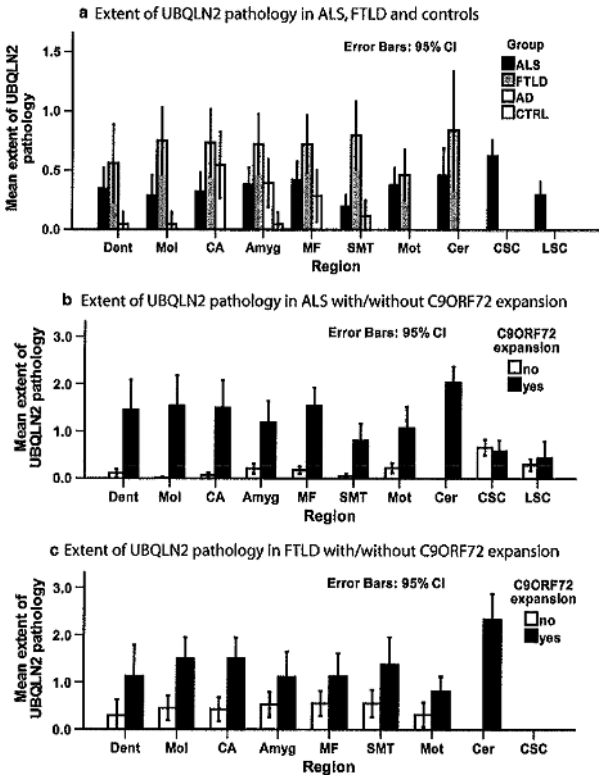

Fig. 4.

Extent of UBQLN pathology in ALS and controls. **a** *Bar plot* shows mean extent of UBQLN pathology in different regions of the central nervous system in ALS, FTLD-TDP and CTRL, **b** *Bar plot* shows mean extent of UBQLN pathology in different regions of the CNS in ALS cases with/without *C9ORF72* expansions, **c** *Bar plot* shows mean extent of UBQLN pathology in different regions of the CNS in FTLD-TDP case with/without *C9ORF72* expansions. *Whiskers* in *bar plot* indicate 95 % confidence interval of mean. *Amyg* amygdala, *CSC* cervical spinal cord, *CA* htppocampal CA regions/subiculum, *Cer* cerebellum, *Dent* hippocampal dentate gyrus, *LSC* lumbar spinal cord, *MF* middle frontal gyrus, *Mol* hippocampal molecular layer, *Mol* motor cortex, *SMT* superior or midtemporal gyrus

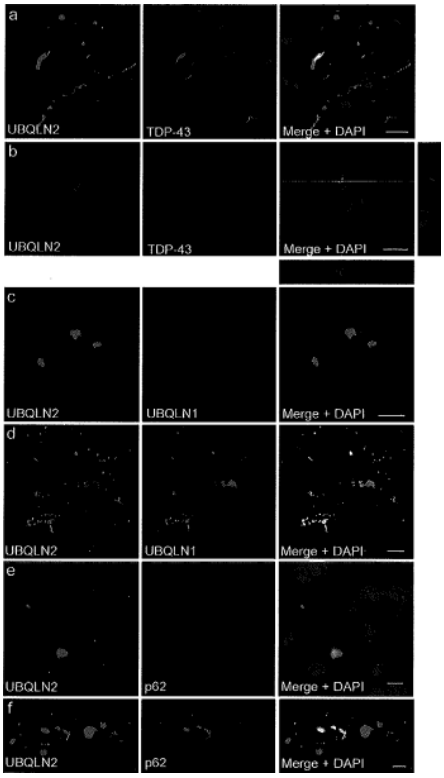

Fig. 5.

Relation of UBQLN pathology to UBQLN1, TDP-43 and p62. Double-labeling IF analyzed by confocal microscopy shows **a** hippocampal dystrophic neurites and aggregate-like formations as detected in ALS/FTLD-TDP with *C9ORF72* expansion to show a partial co-localization of immunoreactivity for UBQLN and TDP-43 in the aggregate-like alteration, **b** Higher resolution image shows UBQLN pathology to be surrounded by TDP-43 immunoreactive material. **c** Hippocampal dentate cytoplasmic neuronal inclusions show immunoreacfivity with a general UBQLN antibody, but not for UBQLN1, **d** In contrast, hippocampal molecular layer aggregate-like formations show immunoreactivity for general UBQLN and UBQLN1. **e** Cerebellar granular layer cytoplasmic neuronal inclusions as detected in ALS/FTLD-TDP case with *C9ORF72* expansions partially show immunoreactivity for UBQLN and p62. **f** Hippocampal molecular layer aggregate-like formations only partially show immunoreactivity for both UBQLN and p62, though UBQLN pathology is more extensive than p62 pathology. *Scale bars* **a**, **c**, **f** 10 μm; **b**, **e** 5 μm; **d** 20 μm

Table 1

Demographic and clinical data of ALS, FTLD-TDP and control autopsy cases

	ALS all	ALS C9+	ALS C9−	FTLD-TDP all	FTLD-TDP C9+	FTLD-TDP C9−	AD	CTRL
N (female/male)	75 (30/45)	14 (5/9)	61 (25/36)	30 (10/8)	9	21	14 (9/5)	11 (5/6)
Median (interquartile range)								
Age (years)	61 (55–69)	58 (55–62)	65 (56–74)	70 (63–76)	65 (54–75)	70 (63–77)	83 (78–85)	68 (60–78)
Duration (years)	2 (1.2–4)	2 (1–2)	2 (1.3–4)	6 (4.3–10)	7 (5–9)	7 (4–11)	12 (9–13)	
ALSFRS-R	19 (15–25)	23 (17–32)	11 (6–17)					
MRCS	38 (34–45)	40 (34–45)	41 (35–48)					
F-words	10.5 (8–13)	11 (8–15)	10 (7–13)	3 (2–10)				
Oral trail	51 (50–52)	51 (48–52)	51 (50–52)	25 (23–27)				
FBI	45 (32–53)	42 (36–50)	36 (30–55)	34 (12–56)				
MMSE	30 (28–30)	30 (29–30)	30 (27–30)	20 (11–27)			8 (2–15)	
Brain weight (kg)	1.35 (1.12–1.43)	1.33 (1.24–1.4)	1.32 (1.22–1.43)	1.06 (1.0–1.28)	1.13 (1.0–1.3)	1.1 (1.0–1.2)	1.06 (0.9–1.14)	1.33 (1.23–1.3)

Data for the neuropsychological tests was not available for FTLD-TDP C9+

Age age at death, *ALSFRS-R* ALS functional rating scale, *C9+* with C9ORF72 hexanucleotide repeat expansion, *C9−* without C9ORF72 hexanucleotide repeat expansion, *duration* duration of disease, *F-words* F-words test, *FBI* frontal behavioral inventory, *Oral trail* Oral trail-making test, *MMSE* Mini-Mental State Examination. *MRCS* Medical Research Council Sumscore