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RESEARCH ARTICLE

Cognitive decline in amyotrophic lateral sclerosis: Neuropathological substrate and genetic determinants

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Abstract

Cognitive impairment and behavioral changes in amyotrophic lateral sclerosis (ALS) are now recognized as part of the disease. Whether it is solely related to the extent of TDP-43 pathology is currently unclear. We aim to evaluate the influence of age, genetics, neuropathological features, and concomitant pathologies on cognitive impairment in ALS patients. We analyzed a postmortem series of 104 ALS patients and retrospectively reviewed clinical and neuropathological data. We assessed the burden and extent of concomitant pathologies, the role of *APOE* ε4 and mutations, and correlated these findings with cognitive status. We performed a logistic regression model to identify which pathologies are related to cognitive impairment. Cognitive decline was recorded in 38.5% of the subjects. Neuropathological features of frontotemporal lobar degeneration

Ricard Rojas-García and Ellen Gelpi are contributed equally to this work.

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(FTLD) were found in 32.7%, explaining most, but not all, cases with cognitive impairment. Extent of TDP-43 pathology and the presence of hippocampal sclerosis were associated with cognitive impairment. Mutation carriers presented a higher burden of TDP-43 pathology and FTLD more frequently than sporadic cases. Most cases (89.4%) presented some degree of concomitant pathologies. The presence of concomitant pathologies was associated with older age at death. FTLD, but also Alzheimer's disease, were the predominant underlying pathologies explaining the cognitive impairment in ALS patients. In sum, FTLD explained the presence of cognitive decline in most but not all ALS cases, while other non-FTLD related findings can influence the cognitive status, particularly in older age groups.

KEYWORDS
Alzheimer's disease, amyotrophic lateral sclerosis, ALS-FTD, frontotemporal dementia, neuropathology, TDP-43 protein

1 | INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by progressive weakness and atrophy because of loss of motor neurons. Cognitive decline and behavioral impairment have been recently recognized as part of the disease (1,2). Approximately half of the patients with ALS have cognitive or behavioral impairment and around 10–20% fulfill clinical diagnostic criteria of any of the clinical variants of frontotemporal dementia (FTD), mainly behavioral variant (bvFTD), but also of primary progressive aphasia (PPA) (2–5). The majority of ALS cases and almost half of FTD patients harbor neuronal, and a proportion also glial, inclusions of TAR DNA-binding protein 43 (TDP-43) at neuropathological examination. In addition, both diseases share genetic alterations such as the hexanucleotide expansion in *chromosome 9 open reading frame 72* (*C9orf72*) gene, as well as mutations in the *tank-binding kinase 1* (*TBK1), sequestosome-1 (SQSTM1), TAR DNA-binding protein* (*TARDBP*), or *valosin-containing protein* (*VCP)*. For these reasons, ALS and FTD are now recognized as a disease continuum (6). FTLD is the pathological substrate underlying most FTD cases. The term frontotemporal lobar degeneration (FTLD) is used as an umbrella term for the different clinical variants (bvFTD, semantic, and non-fluent PPA) as well as a neuropathological term that refers to neuronal loss and gliosis of the frontal and temporal lobes. As a matter of clarity, we refer to the neuropathological concept when using the term FTLD throughout the text.

On the other hand, concomitant neurodegenerative disease-related proteinopathies are frequent and have been primarily associated with age at death (7–9). It remains unclear whether this overlap is coincidental or driven by common physiopathological mechanisms leading to global protein misfolding and aggregation. Genetic factors, especially the presence of *apolipoprotein E* (*APOE*) ε4 allele, an extensively studied potential genetic risk in Alzheimer's disease (AD), has been associated with the presence of AD-related co-pathologies (9–11). Previous work suggests that *APOE* haplotype also influences the risk of developing hippocampal sclerosis (HS) with TDP-43 proteinopathy in aging (12,13), as well as FTD in patients with ALS (14,15). Whether cognitive impairment in ALS patients is related to underlying FTLD or because of another concomitant pathology remains unclear.

The purpose of this study was to evaluate the influence of demographic, genetic, and pathologic features, including the presence of concomitant pathologies, on cognitive impairment in a neuropathologically defined cohort of ALS-TDP patients.

2 | METHODS

2.1 | Standard protocol approvals, registrations, and patient consents

All cases meeting criteria for a primary clinicopathological diagnosis of ALS associated with TDP-43 proteinopathy were selected from the Neurological Tissue Bank (NTB), Hospital Clínic - IDIBAPS Biobank in Barcelona from the period 1994 to 2018. In order to get a homogeneous sample, patients without TDP-43 pathology and mimics were excluded. The study was approved by the Ethics Committee of Hospital Clínic de Barcelona. All individuals or relatives had given their informed consent for the use of brain tissue for research.

2.2 | Clinical classification

We retrospectively reviewed the medical records available at the NTB and contacted the neurologists who attended the patients during life. As we included ALS donors since 1994, some cases had not been systematically screened for cognitive impairment. In those subjects, the presence and the grade of cognitive impairment were assessed by the global impression of the treating neurologist. They were asked to fill in a form that included the following clinical information: age at onset, age at death, disease duration, site of onset (bulbar/spinal), presence or absence of cognitive or behavioral impairment, and diagnosis of bvFTD or PPA. The severity of the cognitive/behavioral impairment was retrospectively assessed according the CDR® Dementia Staging Instrument plus National Alzheimer's Coordinating Center Behaviour and Language Domains (CDR plus NACC-FTLD) (16). This rating scale classifies the severity of the dementia according eight domains in: no cognitive impairment (score of 0), mild cognitive impairment (score of 0.5) or mild, moderate or severe dementia (scores of 1, 2, and 3, respectively). The cognitive domains affected were assigned according to the expert opinion of the attending neurologist or the neuropsychological evaluation. For every patient, we considered the last information available before death. Cases with insufficient clinical information to be classified were excluded from the study. With this information, patients were classified into three clinical subtypes: i) ALS cognitively not impaired (ALSni) when cognitive decline was not present or reported, ii) ALS with cognitive impairment (ALSci) when cognitive or behavioral decline were reported but without fulfilling bvFTD or PPA criteria and iii) ALS with frontotemporal dementia (ALS-FTD) when patients fulfilled criteria for bvFTD or PPA (2,17,18).

2.3 | Neuropathological work-up

Neuropathological examination was performed according to standardized protocols at the NTB. Half brain was dissected in the fresh state, frozen and stored at −80°C and the other half was fixed in formaldehyde solution for 3 weeks. At least 25 representative brain areas were embedded in paraffin, cut at 5 μm and stained with hematoxylin & eosin and luxol fast blue in selected brain areas. Immunohistochemistry was performed using various antibodies including anti-βA4, anti-pTau, anti-RD3 and anti-RD4 Tau, anti-α-synuclein, anti-ubiquitin, anti-p62, anti α-internexin, anti-FUS, and anti-TDP-43, and phTDP-43. Immunoreaction was visualized by the EnVision + system peroxidase procedure (DAKO). Details on tissue section pretreatment, antibody dilution, and incubation time are shown in Table S1.

Disease assessment was performed according to international consensus criteria. All cases were staged following the criteria proposed by Brettschneider for ALS (19). Presence of FTLD was established when neuronal loss and gliosis were observed in temporal and/or frontal regions (20). FTLD-TDP subtype classification was performed based on TDP-43 or pTDP-43 immunohistochemistry following current recommendations (21). HS was considered on HE-stained sections when neuronal loss and gliosis at least in the subiculum and/or CA1 sector of the hippocampus were observed (22). Cases that showed only mild gliosis in subiculum were considered incipient HS. In all cases, we evaluated the presence of fine TDP-43 immunoreactive neurites in the CA1 sector. As previous reports stated that the presence of TDP-43 inclusions in the anterior cingulate may identify patients with clinical bvFTD, we specifically assessed this area in cases with available tissue (23).

For the analysis of concomitant pathologies, we considered the following findings: neurofibrillary pathology, staged according to Braak criteria (24), β-amyloid phases, evaluated according to Thal criteria (25), and neuritic plaque score, assessed according to the Consortium to Establish a Registry for Alzheimer Disease criteria (26). The National Institute on Aging-Alzheimer's Association (NIAA) Guidelines for neuropathologic assessment of AD were applied and a final ABC score was assigned (27). Cerebral amyloid angiopathy was recorded as present or absent. Additionally, possible and definite PART pathology was defined following the current pathological criteria (28). In order to assess whether progressive spinal cord degeneration is associated to astroglial tau pathology, we also evaluated the presence of age-related tau astrogliopathy (ARTAG) at the cervical, dorsal, and lumbosacral spinal level when available and defined thorn-shaped astrocytes and granular fuzzy astrocytes as present/absent, and recorded their location (subependymal, subpial, and perivascular) and the affected level (cervical, dorsal, and/or lumbosacral) (29). Assignments of Lewy body pathology were performed following McKeith criteria (30). Argyrophilic grain disease (AGD) was staged according to the Saito criteria (31). Limbic-predominant age-related TDP-43 encephalopathy (LATE) was evaluated according to the recently described criteria, but only in those subjects without FTLD, as both are TDP-43 proteinopathies and may overlap in temporomedial regions (32). In addition, the presence of granulovacuolar degeneration (GVD) was assessed on HE- and ubiquitin-stained sections in the hippocampus and, if present, was extended to further limbic areas and staged according to Thal et al. (33). Vascular pathology including microvascular lesions and territorial infarcts were recorded as present or absent.

2.4 | Genetic analysis

DNA was extracted from fresh-frozen cerebellum using the QIAamp DNA Mini kit for DNA purification from

tissues (QIAGEN Co.) following the manufacturer's instructions. For *APOE* genotyping two single nucleotide polymorphisms (SNPs) in *APOE* gene were determined (rs429358 and rs7412) using TaqMan genotyping technologies (Life technologies, Carlsbad, USA).

Systematic screening for potential *C9orf72* expansion mutation carriers was performed searching for ubiquitin/p62-positive inclusions in the cerebellum and hippocampus as surrogate and as reported previously (34). The *C9orf72* repeat was confirmed in suspected cases by repeat-primed PCR and fragment-length analysis (35). The identification of other mutations was not performed by systematic screening but by previous studies or usual clinical practice according to the criteria of the treating neurologist (36). Information on other affected family members was not available.

2.5 | Statistical analysis

We compared clinical, genetic, and pathology variables between ALS, ALSci, and ALS-FTD groups with χ^2 or Fisher tests for categorical data and Wilcoxon or Kruskal–Wallis test for ordinal and continuous data. The association of the clinical, demographic, and genetic data with the neuropathological findings was assessed by Kruskal–Wallis test and Fisher test. Finally, we performed a logistic multivariate regression to test the association between cognitive status and neuropathological variables. In this logistic multivariate regression, we included the presence of cognitive decline as dependent variable and the presence of the following neuropathological items as independent variables: frontotemporal lobar degeneration, hippocampal sclerosis, Alzheimer's disease, Lewy body disease, CAA, PART, ARTAG, AGD, GVD, LATE, and vascular pathology. As some of these covariates may suffer from relevant collinearity, other reduced models including less covariates were also assessed. Statistical significance was set at $p < 0.05$ for all analyses.

3 | RESULTS

3.1 | Demographic and genetic features of the ALS cohort

Of the 112 donors with a neuropathological diagnosis of ALS, four were excluded because of the absence of TDP-43 pathology (three ALS-FUS cases and one subject without pathological inclusions). Another four cases were excluded because of incomplete neuropathological exam or insufficient clinical information. Finally, a total of 104 cases (50 male and 54 female) were included in the study. The onset was bulbar in 30.9% of cases. Mean age at onset of motor symptoms was 63.8 years (range 29–87) and the mean age at death was 67.7 (range 34–92).

Cognitive decline was recorded in 40 subjects (38.5%). Thirty-one of them (29.8%) fulfilled diagnostic criteria for bvFTD or PPA (29 and 2 cases, respectively), while nine were classified as ALSci. Table 1 shows demographic, neuropathological, and genetic features of ALSni, ALSci, and ALS-FTD patients. No demographic differences in age at onset, age at death or disease duration were found between ALS, ALSci, and ALS-FTD patients. Bulbar onset was more frequent in subjects with ALS-FTD ($p = 0.03$).

Most patients (67.6%) were homozygous for *APOE* ε3/ε3, 5.1% had ε2/ε3, 22.2% ε3/ε4, and 5.1% ε4/ε4. None of them were homozygous for *APOE* ε2. No significant differences in *APOE* genotypes were found between groups. Mutations in genes reported as causative of or at risk for ALS were found in 21 subjects: 14 *C9orf72*, 2 *TARDBP* (p.A90V and p.I383V), 2 *SQSTM1* (both c.1157C > T), 1 *TBK1* (p.T79del), 1 *VCP* (p.I27V), and 1 *TAF15* (p.G462S). Most of these mutation carriers have been previously reported (34,36,37). More than half of ALS-FTD subjects presented an ALS/FTD related mutation (61.3%), this percentage was higher than in ALSci (0%) and ALSni (3.1%) subjects ($p < 0.001$).

3.2 | Neuropathological features and their relationship with cognitive impairment and genetics

Figure 1 summarizes the main neuropathological features of ALS patients.

3.2.1 | Brettschneider staging (Figure 2A)

The distribution of the TDP-43 pathology according to the Brettschneider score for ALS was as follows: 9 subjects were scored as stage 1, 17 as stage 2, 26 as stage 3, and 51 as stage 4. In one subject the Brettschneider staging was not at all applicable because of the presence of TDP-43 pathology in motor cortex and entorhinal region but not in inferior olive, medullary reticular formation, prefrontal cortex or striatum. The Brettschneider stage was strongly associated with the CDR plus NACC FTLD stage of dementia ($p < 0.01$, Figure 2A). ALS-FTD patients showed significant higher Brettschneider stages than ALSni patients ($p < 0.001$; see Table 1). The presence of a genetic mutation was associated with a higher Brettschneider stage ($p < 0.01$, Figure 2A), while APOE was not. No significant differences concerning disease duration were found between the different Brettschneider stages.

3.2.2 | Hippocampal sclerosis (Figure 2B)

HS was detected in 10 subjects (9.6%). Other eleven cases showed signs of incipient HS. Subjects with ALS-FTD or

TABLE 1 Demographic, neuropathologic, and genetic features in patients ALS, ALSci, and ALS-FTD

	ALSni	ALSci	ALS-FTD	
	n = 64 (61.5%)	n = 9 (8.7%)	n = 31 (29.8%)	*p* value
Demographic features				
Male/female	30/34	4/5	16/15	ns
Age at onset	63.0 (14.3)	67.1 (10.9)	64.3 (11.6)	ns
Mean (SD)				
Age at death	66.6 (13.6)	71.2 (10.3)	68.7 (10.7)	ns
Mean (SD)				
Bulbar onset (%)	23.0%	22.2%	54.2%	<0.05*
CDR plus NACC FTLD	64/0/0/0/0	0/9/0/0/0	0/0/6/9/16	<0.001
0/0.5/1/2/3				
Neuropathologic features				
Brettschneider stage (stage 1/2/3/4)	8/15/21/19	0/1/3/5	1/1/2/27	<0.001*
FTLD (%)	4.7%	44.4%	87.1%	<0.001*,**,***
HS (%)	6.2%	33.3%	41.9%	<0.001*, <0.05**
Genetics				
APOE genotype (%)				
ε2/ε3	6.3%	0 %	3.7%	ns
ε3/ε3	69.8%	55.6%	66.7%	
ε3/ε4	20.6%	22.2%	25.9%	
ε4/ε4	3.2%	22.2%	3.7%	
Mutations (%)				
C9orf72	2 (3.1%)	0 (0%)	12 (38.7%)	<0.001*,***
TARDBP	0 (0%)	0 (0%)	2 (6.5%)	
VCP	0 (0%)	0 (0%)	2 (6.5%)	
TBK1	0 (0%)	0 (0%)	1 (3.2%)	
SQSTM1	0 (0%)	0 (0%)	1 (3.2%)	
TAF15	0 (0%)	0 (0%)	1 (3.2%)	

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSci, amyotrophic lateral sclerosis with cognitive impairment; ALS-FTD, amyotrophic lateral sclerosis with frontotemporal dementia; CDR plus NACC FTLD, Clinical Dementia Rating Dementia Staging Instrument PLUS National Alzheimer's Disease Coordinating Center frontotemporal lobar degeneration Behaviour and Language Domains; FTLD, frontotemporal lobar degeneration; HS, Hippocampal Sclerosis; ns, statistically not significant.

*Significant differences between ALSni and ALS-FTD.

**Significant differences between ALSni and ALSci.

***Significant difference between ALSci and ALS-FTD.

ALSci presented HS more frequently than ALSni cases ($p < 0.01$ and $p < 0.05$, respectively). The presence of HS was also significantly associated with higher stages in the CDR plus NACC FTLD ($p < 0.01$). HS was strongly correlated with Brettschneider staging and presence of FTLD: all subjects with HS presented a stage 4 of Brettschneider, and 88.2% presented also with FTLD. TDP-43-positive neurites in CA1 sector were not detected in any of the cases with HS or incipient HS in the ALSni group. In contrast, two cases with additional AD-type pathology showed frequent fine TDP-43-positive neurites in CA1 sector. The presence of a genetic mutation or the *APOE* ε4 were not associated with a higher prevalence of HS.

3.2.3 | Frontotemporal lobar degeneration (Figure 2C)

Neuropathological features consistent with FTLD were found in 34 subjects (32.7%). Three cases were classified as type A, 25 as type B, 5 showed mixed features of type A and B, and 1 subject had FTLD type C. Most cases with neuropathological FTLD fulfilled clinical criteria of ALS-FTD (77.1%) or ALSci (14.3%). FTLD was strongly associated with higher stages in the CDR plus NACC FTLD ($p < 0.001$). It was present in 87.1% of the ALS-FTD cases, in 44.0% of ALSci, and only in 4.7% of ALSni cases. Moreover, FTLD was present in 63.0% cases with a Brettschneider ALS stage 4 and in

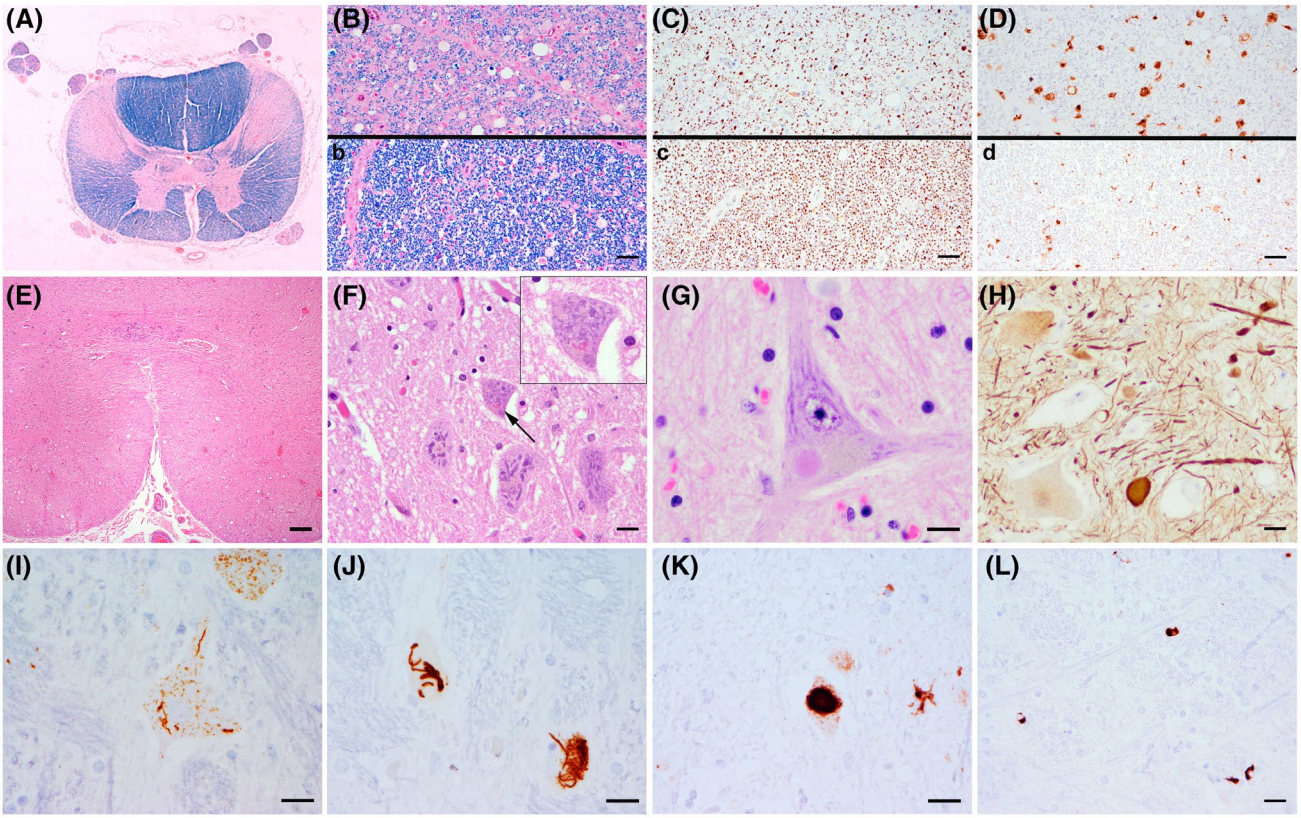

FIGURE 1 **Characteristic neuropathological features of ALS. (A)** Cross section through the thoracic spinal cord shows prominent degeneration of the lateral and anterior corticospinal tract and atrophy of the anterior horns (higher magnification in **E**). There is also myelin pallor in anterior roots compared to posterior roots. The degeneration of the lateral corticospinal tract is characterized by loss of myelin sheaths **(B)**, reduction of axonal profiles **(C),** and increased macrophagic activity **(D)**, as compared with posterior horns **(b, c, d)**. **(F)** some residual motor neurons (here from the n. XII) may contain granular eosinophilic cytoplasmic inclusions or Bunina bodies (arrow and inset). Others may appear as pale spherical inclusions **(G)**. Axonal damage with swellings **(H)** in anterior horns is well depicted by anti-neurofilament immunohistochemistry (large brown structures). **I–L:** Immunohistochemistry for pTDP-43 reveals a spectrum of inclusions including a fine granular pattern in large motor neurons **(I)**, fibrillar and skein like inclusions **(J)**, compact spherical neuronal inclusions **(K)**, and coiled-body like inclusions in oligodendrocytes **(L)**. Scale bars: 20 μm in Bb, Cc, Dd, F; 10 μm in G–L, 100 μm in E

4.3% of cases in stage 3, while it was absent in stages 1 and 2.

ALS subjects with any genetic mutation presented significantly more risk to develop FTLD than ALS without known mutations (80.9% vs. 20.4%, OR 15.92, IC 95% 4.45–73.66, $p < 0.001$). By contrast, *APOE* ε4 was not associated with a higher risk of FTLD or a specific FTLD subtype. Cases carrying the *C9orf72* expansion were neuropathologically classified as subtype B or A/B. We did not find statistically significant differences in the neuropathological subtypes according the APOE genotype.

3.2.4 | Cingulate involvement (Table 2)

Cingulate involvement was present in 70.0% of cases (sparse 16.7%, mild 18.9%, moderate 25.5%, and severe grade in 8.9%). All subjects with FTLD showed, at least, a mild grade of TDP-43 pathology in the anterior cingulate. In contrast, 70% of subjects without FTLD presented none or only sparse pathology in this area.

3.3 | Concomitant pathology

Figure 3 shows some of the representative concomitant pathologies found in ALS patients. Figure 4 shows the proportion of these concomitant pathologies found in our series.

3.3.1 | Presence of concomitant neurodegenerative pathologies (Figure 4A)

Considering all concomitant pathologies together, most (89.4%) ALS patients presented at least one co-pathology at neuropathological examination. Concomitant AD neuropathologic changes, according to "ABC" score, were found in 52 subjects (50.0%) at low, in 8 (7.7%) at intermediate, and in 7 patients (6.7%) at high level. CAA was found in 17 cases (16.3%). Nearly half (48.1%) of the subjects fulfilled criteria for PART pathology (25.0% possible, 23.1% definite). In addition, 38.5% presented ARTAG (18.3% granular

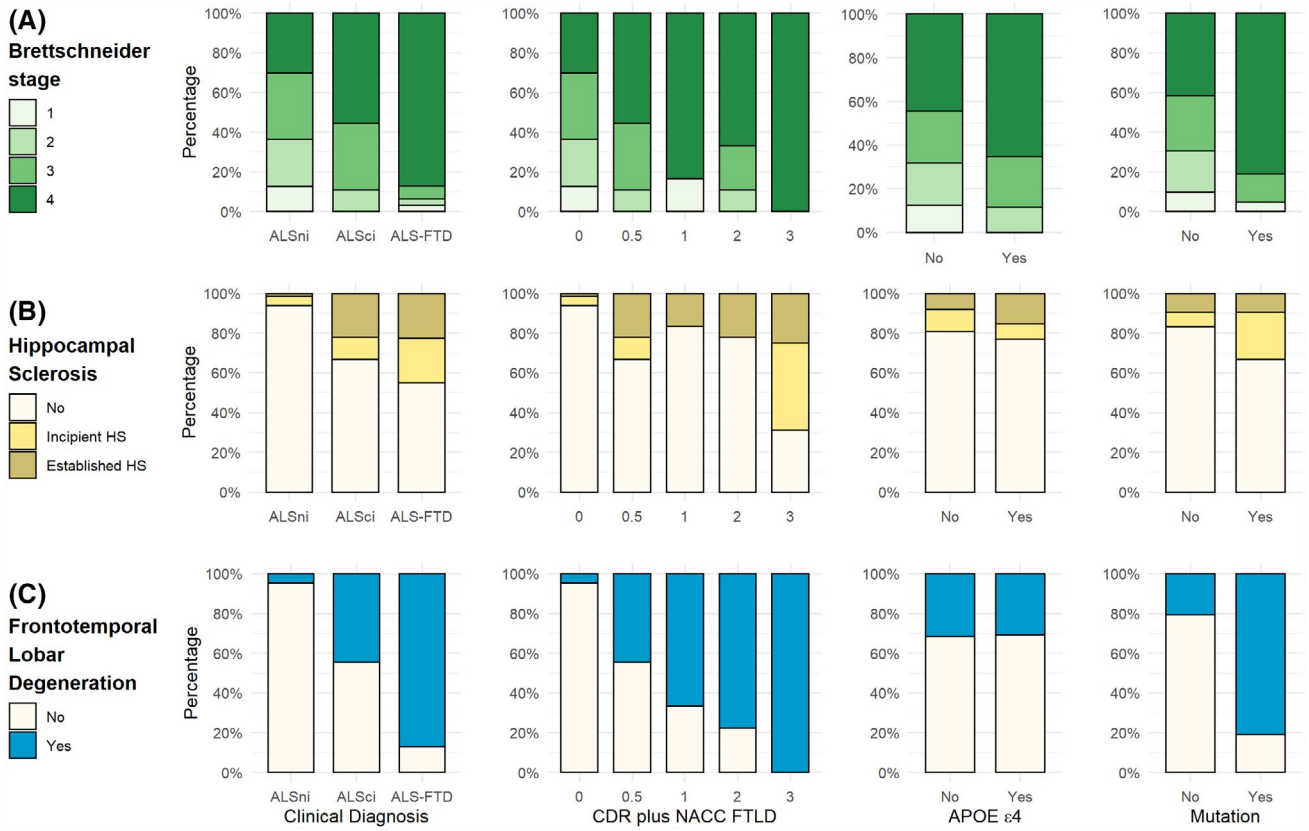

FIGURE 2 Barplots representing the proportion of ALS cases with (A) the different Brettschneider stage, (B) the presence of hippocampal sclerosis and (C) Frontotemporal Lobar degeneration according to their clinical diagnosis, CDR plus NACC FTLD stage, presence of APOE ε4, or presence of mutations in genes reported as causative of or at risk for ALS/FTLD

TABLE 2 Prevalence of TDP-43 pathology in anterior cingulate cortex in ALS spectrum

Presence of TDP-43 in anterior cingulate n (%)	ALSni n = 58	ALSci n = 6	ALS-FTD n = 26
No	25 (43.1%)	1 (16.7%)	1 (3.8%)
Sparse	14 (24.1%)	1 (16.7%)	0 (0%)
Mild	13 (22.4%)	0 (0%)	4 (15.4%)
Moderate	6 (10.3%)	4 (66.7%)	13 (50%)
Frequent	0 (0%)	0 (0%)	8 (30.8%)

Note: n = 90 cases out of 102.

Abbreviations: ALSci, amyotrophic lateral sclerosis with cognitive impairment; ALS-FTD, amyotrophic lateral sclerosis with frontotemporal dementia; ALSni, amyotrophic lateral sclerosis without cognitive impairment.

fuzzy astrocytes, 13.5% thorn-shaped astrocytes, 6.7% both), with 8.3% presenting astroglial pathology also in the spinal cord. ARTAG pathology was found more frequently, but not exclusively, in subjects with AD pathology (44.8% vs. 27.0%, $p < 0.05$). GVD was found in 42.3% of the subjects and was mostly very mild and restricted to the hippocampal subfields CA2/CA1 with only mild entorhinal involvement. GVD was not only strongly related to AD pathology ($p < 0.001$), but also to the presence of FTLD and the *C9orf72* expansion ($p < 0.05$). AGD was detected in 14 subjects (13.5%; 11 subjects at Saito stage 1, 1 subject at stage 2, and 2 subjects at stage 3), while Lewy body pathology was identified in 9.6% (seven subjects restricted to brainstem nuclei, three with limbic involvement, and only one with neocortical involvement). Vascular lesions were identified in 14 subjects (13.5%); 11 of them with small vessel pathology and only 3 of them presenting large vessel infarcts. We observed LATE in 19 (27.1%) of the 70 ALS cases without FTLD.

3.3.2 | Relationship of concomitant pathologies and age at death (Figure 4B)

Concomitant pathologies were more frequent in subjects who died at older age. ABC score for AD was significantly higher in subjects with older age at death ($p < 0.01$). Particularly, Braak and Braak stages for neurofibrillary pathology and amyloid Thal phases were significantly increased in older subjects ($p < 0.01$). The presence of LATE, ARTAG, Lewy body pathology, GVD, and vascular lesions also was significantly higher in older age groups ($p < 0.05$). CAA, PART, and AGD concomitant pathologies did not show significant association with age at death.

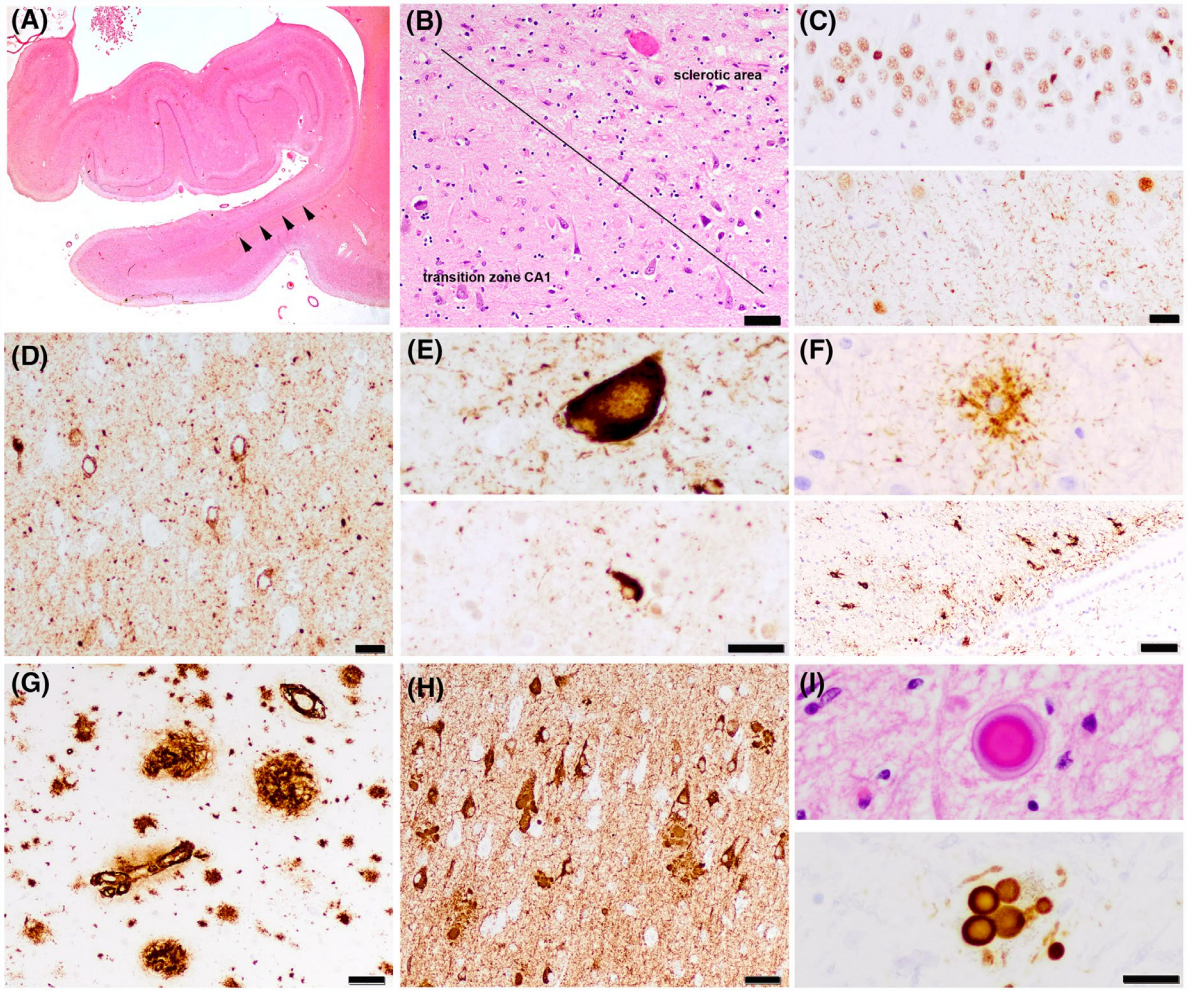

FIGURE 3 **Frequent co-pathologies in ALS. (A–C)** Segmental hippocampal sclerosis is sometimes better identified in more anterior segments of the hippocampus (A, arrowheads). It is characterized by neuronal loss and prominent fibrillar gliosis (B) in the CA1 sector and subiculum. In most cases granule cells of the dentate gyrus harbor TDP-43 protein inclusions in the cytoplasm (C, upper row) and in some cases there are abundant fine threads in the CA1 sector within and adjacent to the sclerotic area (C, lower row). **(D and E)** Argyrophilic grain pathology is well depicted by the AT8 anti-tau antibody (D) and consists of grain-like structures along neuronal processes, abundant threads, and a diffuse cytoplasmic neuronal staining (pretangle type) instead of compact tangles. They are frequently associated with ballooned neurons in the amygdala (E, upper row) and oligodendroglial coiled bodies in temporomedial white matter (E, lower row). Astrocytic tau pathology in form of granular fuzzy astrocytes (F, upper row) and thorn-shaped astrocytes in the glia limitans (F, lower row, here as subependymal and perivascular thorn-shaped astrocyte) are features of age-related tau astrogliopathy (ARTAG). **(G and H)** Alzheimer's disease neuropathologic changes have been also found as co-pathology in a fraction of cases. It is characterized by dense cored amyloid plaques with or without amyloid angiopathy (G) and tau positive neurofibrillary pathology with tangles, neuropil threads, and dystrophic neurites around amyloid deposits (H). **(I)** Lewy body pathology has been detected less frequently and can be encountered in brainstem neurons (HE, upper row, medulla oblongata) and/or limbic system and is well identified with anti-alpha-synuclein antibodies (lower row), where Lewy-neurites are also frequently seen (here in the locus coeruleus). Scale bars: 50 μm in B, F, G, H; 20 μm in C (upper and lower panel), D, E, and I (upper and lower panel)

3.3.3 | Relationship of concomitant pathologies with CDR-NACC FTLD (Figure 4C)

Concomitant pathologies were found more frequently in subjects with reported cognitive impairment (97.5% vs. 84.0%, $p < 0.05$). The CDR-NACC-FTLD score was significantly related to the Braak and Braak stage for neurofibrillary pathology ($p < 0.01$). Subjects with severe stages of dementia also presented more GVD pathology ($p < 0.05$) No significant associations were found between the CDR-NACC-FTLD score and Thal phase of amyloid, CAA, PART, ARTAG, AGD, Lewy body, LATE, or vascular co-pathologies.

3.3.4 | APOE status and concomitant pathologies (Figure 4D)

CAA was found more frequently in subjects with an *APOE* ε4 allele (34.6%, vs. 9.6%; $p < 0.05$). For the rest of the concomitant pathologies, no statistical differences were according to the *APOE* status.

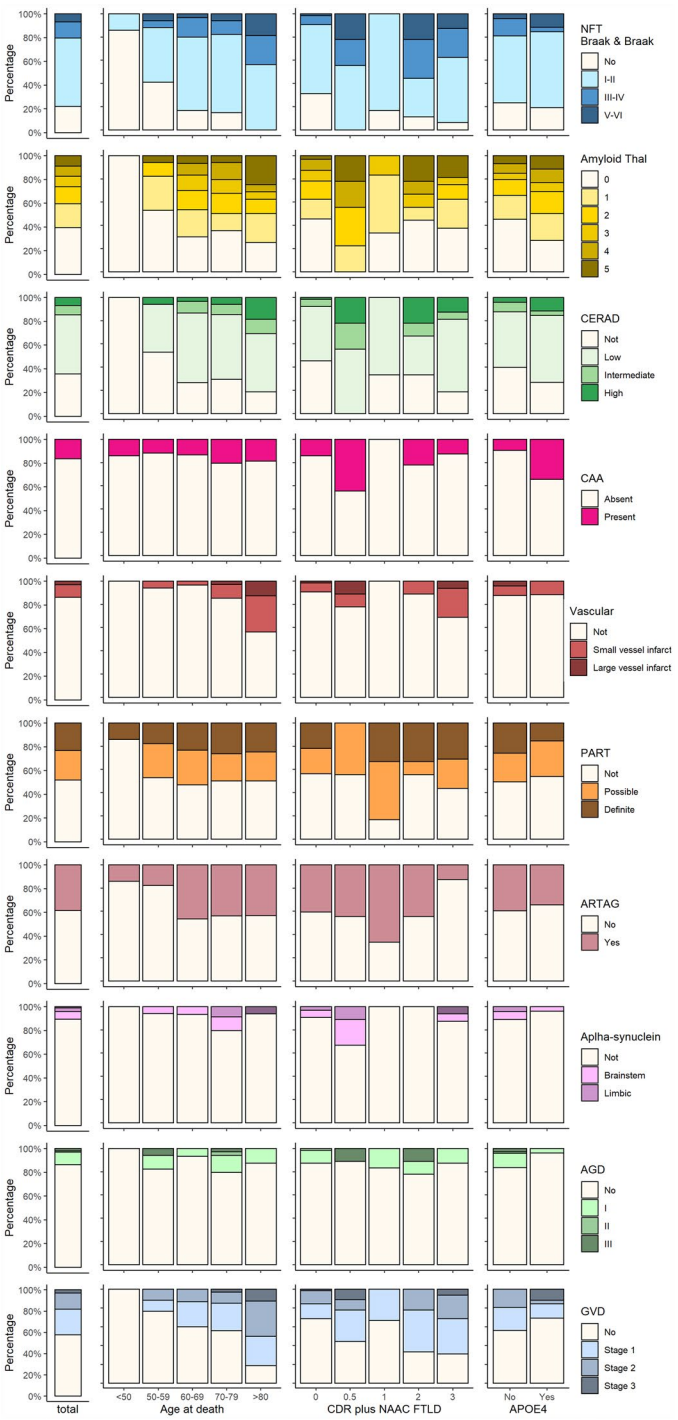

FIGURE 4 **Presence of total concomitant pathologies in ALS cases (A), distribution of pathology according to age at death (B), distribution of pathology according to the CDR NACC plus FTLD staging (C), and distribution of pathology according to the presence of *APOE* ε4 allele (D).** Neurofibrillary tangles were staged following Braak stages (24). Amyloid represents Aβ Thal phases (25). Globally, Alzheimer's disease pathology was staged according the NIA/AA guidelines and an ABC score was assigned (27). Cerebral Amyloid angiopathy (CAA) has been reported as absent or present. Vascular lesions were classified according to small or large vessel pathology. Assignments of Lewy Body pathology were performed following McKeith criteria (30). Possible or definite primary age related tauopathy (PART) was assessed according the current neuropathological criteria (28). Age-related tau astrogliopathy (ARTAG) was evaluated according the current criteria (29). Argyrophilic grain disease (AGD) was staged according to the Saito criteria (31). Granulovacuolar degeneration (GVD) was staged according to Thal et al (33)

3.4 | Determinants of cognitive impairment in ALS

Finally, to assess whether neuropathologic and concomitant pathologies influence the presence of cognitive impairment in ALS patients a logistic regression analysis was performed (Table 3).

Regarding the neuropathological substrate, the presence of FTLD was the strongest determinant for cognitive decline in our model (OR 359.5, 95% CI 20.4–1940.2, $p < 0.001$). We also found that the presence of AD concomitant pathology independently influenced the development of cognitive impairment in ALS patients, especially in those with higher burden of pathology (OR 36.5, 95% CI 3.4–444.2, $p < 0.05$). Other concomitant pathologies did not influence cognitive symptoms in ALS subjects with these analyses. Reduced models considering less covariates in order to avoid collinearity problems showed similar results (Table S2).

Five subjects with reported cognitive impairment (three ALSci and two ALS-FTD) did not present an

TABLE 3 Logistic multivariate regression

Neuropathologic features	Odds ratio (95% CI)	*p* value*
Age at death	0.9 (0.8–1.0)	0.177
Sex (female)	2.7 (0.6–14.5)	0.219
Frontotemporal lobar degeneration	359.5 (20.4–1940.2)	**<0.001**
Hippocampal sclerosis	0.3 (0.1–5.5)	0.459
Alzheimer's disease neuropathological change	36.5 (3.4–444.2)	**<0.05**
Cerebral amyloid angiopathy	2.9 (0.4–20.9)	0.279
α-synuclein	2.3 (0.1–13.7)	0.559
PART	2.3 (0.4–7.1)	0.325
ARTAG	1.9 (0.4–6.5)	0.461
Argyrophilic grain disease	1.0 (0.1–5.5)	0.995
LATE	3.4 (0.3–11.3)	0.315
Granulovacuolar degeneration	1.7 (0.3–8.0)	0.542
Vascular lesions	1.5 (0.1–7.7)	0.748

Note: The dependent (outcome) variable of the model was the presence of cognitive impairment (ALSci and ALS-FTD were considered together). Frontotemporal lobar degeneration was established according to the presence of neuronal loss and gliosis in temporal and/or frontal cortices. Alzheimer's disease neuropathological change was considered as a moderate or high burden of tau and amyloid pathology according the NIA-AA.

Abbreviations: ARTAG, age-related tau astrogliopathy; LATE, limbic-predominant age-related TDP-43 encephalopathy. PART, primary age-related tauopathy.

*p values were obtained from Wald's test, bold indicates statistical significance.

unequivocal neuropathological substrate explaining their symptoms. This situation was found more frequently in the ALSci group than in the ALS-FTD group (33.3% vs. 6.5%; $p < 0.05$). The two subjects fulfilling criteria for ALS-FTD but lacking an obvious neuropathological substrate presented genes reported as pathogenic of or at risk for ALS (1 *TARDBP* and 1 *VCP*). The *TARDBP* carrier presented frequent TDP-43 inclusions in amygdala with focal gliosis and neuronal loss. The *VCP* carrier, by contrast, had very few TDP-43 inclusions restricted to motor neurons (Brettschneider stage I), but presented concomitant AGD pathology (Saito I).

4 | DISCUSSION

In this work, we have analyzed the potential effects of the extent of TDP-43 pathology, concomitant pathologies, and genetic variables on the cognitive-behavioral status in a large series of neuropathologically confirmed ALS patients.

Though the growing evidence of ALS and FTLD overlap, only few neuropathological series have been reported so far (9,38). In our series of neuropathologically confirmed ALS patients, cognitive impairment was reported in more than one-third (38.5%) of cases. Most of them showed a pattern of FTLD at neuropathology. Neither the Brettschneider stage nor the presence of FTLD were associated with sex, age at death or disease duration. Spencer et al. recently reported that patients with long-duration ALS (>10 years) showed less frequent TDP-43 pathology and less severe lower motor neuron involvement. In our series, only six patients had a disease duration of 10 years or more and two of them had no TDP-43 but FUS pathology (39). However, those harboring TDP-43 pathology had no different TDP-43 severity stages (as assessed by Brettschneider staging) compared to the standard duration group in their study (40). This was also observed in our series. Also in accordance with previous studies, the presence of a mutation was the strongest determinant for comorbid ALS and FTLD (14), even in the absence of family history within the ALS/FTD spectrum (36). Bulbar onset was more frequent in subjects with ALS-FTD, a finding that also has been found in other studies (37,41).

We found that an FTLD neuropathological pattern and/or HS are the most frequent neuropathologic substrate of cognitive impairment in ALS subjects. Neither the Brettschneider stage nor the presence of FTLD or HS were associated with disease duration (40). At the same time, the presence of cognitive impairment was associated with a Brettschneider ALS stage 4 and the presence of TDP-43 pathology in anterior cingulate. In such cases, showing extensive ALS and FTLD-related TDP-43 proteinopathy it might be useful either to apply both proposed staging systems in parallel, that is, that for ALS-TDP and that bvFTD with TDP-43 inclusions, or even to combine both and propose a new staging fusing both particularly when one or the other system fails to appropriately classify the pathology. One of the caveats of Brettschneider ALS stages is that the topography alone may not explain cognitive symptoms. Previous studies have suggested that the burden of TDP-43 pathology in the anterior cingulate cortex discriminates cases with clinical bvFTD (23). In our work, almost all subjects diagnosed as ALS-FTD showed at least mild TDP-43 pathology in the anterior cingulate, and this was also present in 32.7% of subjects in the ALSni group. This finding suggests that the cingulate burden of TDP-43 could be more sensitive than specific to discriminate cognitive involvement in ALS patients. However, because of the retrospective methodology of our study, it is difficult to elucidate if subtle cognitive alterations might have been underdiagnosed in these subjects, especially in the last stages of the disease.

Moreover, in HS the presence of TDP-43 proteinopathy is a frequent finding, independently of motor neuron involvement. In ALS cases with HS, the application of TDP-43-stages according to Brettschneider's proposal might result in stage 4 although hippocampal involvement by TDP-43 would be more likely related to HS and not necessarily to a sequential progression of pathology from motor regions. Therefore, the staging systems for ALS, FTLD, and HS, when appearing concomitantly, should be critically rethought. This also applies to the

recently described "LATE," which by itself may also contribute to cognitive decline. It should be considered a separated entity from FTLD, as it manifests with a different phenotype, it occurs particularly in older age groups (the "oldest old," >85 years) and has a relatively restricted neuroanatomical distribution (32). In the context of ALS-TDP, whether the presence of TDP-43 pathology in the limbic system represents LATE pathology, or whether it is a restricted form of FTLD, remains a matter of discussion.

Our study also reveals a high frequency of concomitant pathologies in ALS patients, however, at different severity grades. It is currently well known that the accumulation of brain pathologies is common and that these may influence the cognitive state or lower the threshold for the development of cognitive decline (7,8). The frequencies of concomitant pathologies found in our series are in accordance with those reported in previous works (9,38). Most ALS patients presented any grade of tau pathology in the form of neurofibrillary tangles: PART pathology was found in approximately half of the cases; around one-third to one-half also presented amyloid-β pathology; and about a 10th presented any grade of α-synuclein pathology. Our study also evaluated the presence of ARTAG pathology in the brain and spinal cord of ALS subjects, as it represents the most frequent astrocytic tau pathology found in aging brains. We found that more than a third cases (38.5%) presented ARTAG pathology. In a few subjects, ARTAG pathology was also found in the spinal cord, particularly in older age groups. Whether the spinal astroglial pathology may be elicited by the chronic neurodegenerative process or not remains a matter of further studies.

In addition, we found a quite considerable prevalence of AGD co-pathology in our series (15.5%). AGD is a common sporadic neurodegenerative disease of old age but is rarely seen in young subjects. Of note, some of the subjects with AGD pathology were relatively young (mean age at death 70.9 years, range 58–84). Previous reports point that AGD and TDP-43 are occasionally concurrent in ALS/FTLD cases (42,43). These findings may strengthen the hypothesis of the existence of a link between TDP-43 pathology and argyrophilic grains. However, the pathogenesis of this potential interaction remains unclear.

GVD, a feature observed in cellular stress conditions and some neurodegenerative pathologies including AD was not a frequent feature and, if present, was mostly very mild and restricted to the hippocampal subfields CA2/CA1 with only mild entorhinal involvement (33). GVD was not only more frequently related to AD pathology, including both, neurofibrillary tangles and amyloid plaques, but was also observed more frequently in individuals with FTLD pathology or in those carrying the *C9orf72* expansion. An association between GVD and TDP-43 pathologies has been recently reported. It has been suggested to be potentially triggered by TDP-43 in the hippocampus and has been linked to a cell death mechanism (44,45). GVD could, therefore, represent an additional, TDP-43-related mechanism leading to cognitive impairment in ALS besides its relationship with Alzheimer's disease neuropathological change.

In our series the strongest determinant for the presence of concomitant pathologies was the age at death: while subjects aged 60 or less rarely had co-pathologies, those >60 years had at least 1, and those >70 years had 2 or more co-pathologies. Although it is not possible to further elucidate this observation given the absence of a matched control group in our study, this frequency of co-pathologies seems to appear at relatively early ages in ALS cases. Moreover, subjects with reported cognitive impairment presented with more severe AD pathology.

The presence of the *APOE* ε4 allele is a well-known risk factor to develop AD. For other dementias, this relationship is not well established. Chio et al. found that the presence of the allele ε2 significantly increased the risk of cognitive impairment in ALS patients (14). Other studies, including two meta-analyses, found a relationship between FTLD and the presence of the ε4 allele (15,46). These studies were limited, as they were not performed in a pathologically confirmed cohort or in ALS patients. Recently, Yang et al. found an age-dependent effect of *APOE* ε4 on TDP-43 and HS, (13) and Wennberg et al. on TDP-43 deposition independently of Aß in AD pathology in a large postmortem series (12). In our postmortem study, we also analyzed the effect of *APOE* on ALS and cognition, but we did not find statistically significant associations between *APOE* ε4 allele and the presence of FTLD, FTLD subtypes or HS. Except for CAA, we neither found differences in the presence of concomitant pathologies in *APOE* ε4 carriers. Because of the low frequency of the *APOE* ε2 allele observed in our series, an association with the presence or absence of FTLD cannot be established.

When evaluating the clinical impact of neuropathological findings, we found that the presence of FTLD is, by large, the predominant underlying pathology that better explained the cognitive impairment in ALS patients. However, our analysis reveals that AD also can influence the development of cognitive symptoms in these patients. In addition, we found a few cases with reported cognitive decline but without FTLD or enough AD pathology to justify cognitive impairment. The two subjects with ALS-FTD diagnosis lacking an obvious neuropathological substrate for cognitive impairment were carriers of mutations in genes reported as pathogenic of or at risk for ALS (1 *TARDBP* and 1 *VCP*). The *TARDBP* mutation carrier showed TDP-43 inclusions in amygdala associated with focal gliosis and neuronal loss, while the *VCP* mutation carrier showed mild argyrophilic grain pathology with ballooned neurons. The involvement of the amygdala in behavioral disturbances is well known (47). Therefore, despite not having a classical FTLD-pattern, the presence of focal amygdala

degeneration could have been sufficient explanation for the cognitive impairment. So finally, in three ALS cases in which cognitive impairment was reported, we found no adequate neuropathological explanation. Given the retrospective nature of the study, we cannot draw any conclusion concerning this finding. However, it may be an interesting focus of interest in future studies with precisely phenotyped series.

Our study has several limitations. First, due the retrospective nature of the study, which included cases that had been evaluated years before the acceptance of the concept of the ALS-FTLD continuum, two-thirds of the subjects had not been systematically screened with a formal neuropsychological evaluation, cognition was assessed by the global impression of their neurologist. While this might underestimate the presence of cognitive impairment particularly when it was subtle or in the last stages of the motor neuron disease, we still found a good association between the cognitive information reported by the neurologist and the neuropathological examination. In that sense, further prospective studies with accurate screening tools for cognitive decline are needed to validate the conclusions of this work. Second, as a single Brain Bank series, it is possible that our results do not represent the whole ALS population. Therefore, some clinical or genetic features may be overrepresented because of a selection bias for brain donation. Finally, while a morphological screening for *C9orf72* was performed in all cases, genetic tests for other mutations have not been performed systematically (34).

5 | CONCLUSIONS

Cognitive impairment is a frequent finding in patients with ALS. Most, but not all cases, present with FTLD with or without hippocampal sclerosis as the underlying pathology, have higher ALS-Brettschneider stages and higher burden of TDP-43 pathology in the anterior cingulate. AD can also influence the development of cognitive symptoms in ALS patients. The presence of a mutation, but not the *APOE* genotype, is a strong determinant for the presence of cognitive decline. Moreover, concomitant neurodegenerative pathologies, particularly AD, are more frequent in older ALS subjects with cognitive decline, while AGD may appear earlier than in non-ALS patients.

These findings contribute to broaden the knowledge on the overlap between ALS and FTD and emphasize the importance of an accurate assessment of non-motor features in ALS patients including clinical, neuropsychological, neuroimaging, and biofluid biomarkers through an integrative approach. Well-characterized patients will undoubtedly profit from better directed therapeutic interventions and care.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

JTS, MB, RSV, AL, MP, MAR, JG, AC, MPC, LB, JS, and RRG collected clinical data. SBE, IA, LMP, and EG collected neuropathological data. TX, AA, and JC performed genetic analysis. SBE drafted and prepared the manuscript. SBE and MB performed statistical analysis. RRG and EG conceived the study and its design. All authors read, revised, and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.
Supplementary Material

TABLE S1 Antibodies used for immunohistochemistry and their pretreatments
TABLE S2 Reduced model of logistic regression: The dependent (outcome) variable of the model was the presence of cognitive impairment (ALSci and ALS-FTD were considered together). The reduced model avoids covariates with collinearity problems

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