An Assessment of Possible Neuropathology and Clinical Relationships in 46 Sporadic Amyotrophic Lateral Sclerosis Patient Autopsies

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Key Words

Amyotrophic lateral sclerosis · Amyloid β protein · Atrophy · Frontotemporal dementia · Frontotemporal lobar degeneration · Motor neuron diseases · Neurofibrillary tangles · Tau · Parkinson's disease

Abstract

Background: Recent studies have suggested overlapping pathological features among motor neuron, cognitive and neurodegenerative diseases. **Aims/Methods:** Secondary analysis of 46 amyotrophic lateral sclerosis (ALS) patient autopsies was performed to independently assess pathological feature prevalence (e.g. percent of patients with any positive finding), degree of severity (e.g. mild, moderate, severe), and 2,200+ potential clinical/neuropathological correlations. The possible impact of gender, onset age, onset type (limb vs. bulbar), riluzole treatment, and severe TDP-43 pathology was assessed within patient subgroups. **Results:** Assessed features (prevalence, severity) include: lateral corticospinal tract degeneration (89%, moderate); Purkinje cell loss (85%, mild); localized neuronal loss (83%, mild to moderate); TDP-43 inclusions (80%, moderate); Betz cell loss (76%, mild); neurofibrillary tangles (78%, severe); anterior corticospinal tract degeneration (72%, moderate); spinal ventral root atro-

phy (65%, moderate); atherosclerosis (35%, mild); β -amyloid (35%, mild); tauopathy/tau inclusions (17%, mild); ventricular dilation (13%, mild); Lewy body formation (11%, mild); microinfarcts (7%, mild); and α -synuclein (4%, mild). Twenty-two percent of patients met criteria for Alzheimer's disease (AD) and 26% for frontotemporal lobar degeneration. Substantial differences were identified in the AD group and in the different onset age groups. **Conclusion:** Our findings support the hypothesis that ALS and its variants could comprise a larger neuropathological continuum.

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Introduction

In recent years, investigations into the pathophysiology of amyotrophic lateral sclerosis (ALS) have led to suggestions that ALS lies on a pathological continuum with frontotemporal dementia (FTD) [1, 2]. ALS is a disease of the motor neurons most typically characterized by muscle paralysis resulting from the loss of motor neurons in the spinal cord, brainstem, and motor cortex [3]. Nonetheless, mild to moderate cognitive deficits are present in about 45% of the ALS patient population and about 6% develop FTD [4].

We hypothesize that a neuropathological continuum could exist that comprises a larger expanse of diseases, including but not limited to: ALS, primary lateral sclerosis, Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD), FTD, Parkinson's disease (PD), Pick's disease, among others. The implication that ALS is directly related to diseases whose symptoms comprise, in part, cognitive decline and/or behavioral aberrations, is a departure from the more traditional thought [5], which ascribes to ALS only the aforementioned physical detriment [6]. In addition to the known motor, cognitive, and behavioral impairments, autonomic and homeostatic disturbances have also been reported [7, 8]. Moreover, recent work in the field does indicate that ALS and its variants, such as the Guamanian ALS-PD disorder, could potentially exist within a possible etiological spectrum or continuum of multiple neuropathologies [1, 2, 9].

In both clinical and animal model studies, it has become increasingly apparent that many pathology features are shared between various neurodegenerative diseases as well as nonpathological general aging. Such features include protein aggregate inclusions consisting of amyloid β [10] and/or TDP-43 [11, 12], Lewy bodies [13, 14], and neurofibrillary tangles (NFTs) [15]; neuronal losses and degeneration to specific areas of the brain and spinal cord [3]; circulatory changes of cerebrospinal fluid (CSF) or hemodynamics (blood flow) [16], and neural atrophy, particularly in the spinal ventral roots, paraspinal fiber, and brain [17]. Such common pathology features could give way to seemingly disparate but yet shared pathological etiologies manifested as a spectrum of different disease phenotypes.

In this exploratory study, we assess possible relationships between clinical and neuropathological features from the autopsies of 46 patients with sporadic ALS. We independently assess the prevalence, severity, and correlations between a host of ALS-specific as well as general neuropathology measures in varying locations of the brain and spinal cord, including cellular pathological markers, circulatory and hemodynamic measures, corticospinal tract degeneration, neural atrophy, neuronal losses of specific cell types and locations, NFTs by location, and pathological postmortem diagnosis of comorbid AD or FTLD. The goal of this study was to identify potentially compelling common pathological features or relationships among this ALS study population that could be statistically evaluated in larger, future studies or compared to other neurodegenerative diseases. In addition to assessing the overall ALS study population, we also examine subgroups of patients to evaluate the possible impact

of gender, comorbid AD, ALS onset age, ALS disease duration, ALS onset type (i.e. limb vs. bulbar), continuous riluzole treatment, and 'severe' TDP-43 pathology, on the observed neuropathology features.

Methods

We performed a secondary analysis of autopsy and histological reports completed by a single neuropathologist at Emory ALS Clinic (Emory University Hospital, Atlanta, Ga., USA). All post-mortem examinations were performed over a period of approximately 10 years. Consent to perform each autopsy for the purpose of research was provided by the patient's family. Autopsy was performed of the brain and spinal cord for 51 patients of whom 46 patients were included in this study. Examination included a multitude of gross and microscopic parameters as described in table 1. The internal review boards of Emory University and Georgia Institute of Technology approved this study.

Inclusion Criteria

Inclusion criteria consisted of the following: (1) patients for which the full battery of pathology and immunohistochemistry tests were successfully conducted (see autopsy procedure); (2) patients with sporadic ALS for which available genetic testing (SOD1, ALS2 gene, etc.) or familial history were negative – note that given the timeline of autopsies, not all genetic tests (C9ORF72, FUS, TARDBP, etc.) were available for all patients; and (3) patients for which complete clinical records were available, including birth-date, sex, first onset symptom date and location, exclusionary diagnostic tests (MRI, CSF, electrophysiology, etc.), riluzole prescription usage history, and genetic testing or complete familial history. These criteria resulted in a total of 46 of the 51 patients being included in the study results.

Autopsy Procedure

The brain and spinal cord of each patient was removed and weighed before a sagittal cut. The left half was utilized for the present investigation while the right was frozen for future studies. The brain was cut in serial coronal section at 0.5-cm interval. Initially, gross examination of the slices was performed examining for pigmentation, atherosclerosis, and visual neurodegeneration. Neuropathology cassette included: hippocampus; amygdala and frontal cortex; parietal and occipital cortex; anterior basal ganglia and caudate; mid basal ganglia and insula; perirolandic cortex; thalamus and hypothalamus; midbrain; pons and cerebellum; medulla and cervical cord; upper cord; lower cord; dorsal root ganglia and dorsal roots, and ventral roots. Tissue was fixed in 4–8% paraformaldehyde. Briefly, key immunohistochemistry included: hematoxylin and eosin staining; modified Bielschowsky silver method; TDP-43 antibody; tau antibody; amyloid β antibody; α -synuclein antibody. Pathological feature severity is parametrically determined by extent of staining (see Analysis).

Clinical Parameters

Assessed clinical parameters included ALS symptom onset age, onset type, diagnostic delay (the amount of time elapsed between the onset of the first symptom of ALS and diagnosis of ALS by a neurologist), disease duration (the amount of time elapsed be-

Table 1. Description of assessed pathology features

Pathology feature	Description/reason for inclusion
β -Amyloid	The presence of excess β -amyloid protein, typically in tangles or inclusions, is a known feature of AD. However, β -amyloid changes have also been seen in the CSF of ALS patients [10].
Lewy body formation	α -Synuclein immunohistochemistry is used to identify Lewy bodies, which are abnormal aggregates of protein present in PD, Lewy body disease, and other disorders, including some cases of ALS [13, 14].
Tauopathy	The presence of tau protein aggregates is a known feature of AD and FTD. The presence of such aggregates in ALS is presently controversial [45, 47–52].
TDP-43 inclusions	TDP-43, or transactive response DNA-binding protein 43 kDa, is a common pathological feature of both FTD/FTLD and ALS [11, 12]. Cytoplasmic inclusions particularly in the anterior horn are a common feature of ALS. TDP-43 has been shown to affect neuronal activity response factor in the dendrites of hippocampal neurons, suggesting possible roles in regulating mRNA stability, transport and local translation in neurons [53, 54].
NFTs	Primarily comprising hyperphosphorylated tau proteins, NFTs are, along with senile plaques, pathological features of AD. Quantity of NFTs is thought to be related to the degree of dementia, suggesting that their accumulation is possibly related to neuron dysfunction [29, 55]. They have been found in ALS populations in the hippocampus [15].
Atherosclerosis	A specific form of arteriosclerosis in which an artery wall thickens as a result of invasion and accumulation of white blood cells. It has been proposed that ALS could potentially be caused by constrictions in veins draining the spinal cord and brain or from inflammatory disease.
Infarcts/microinfarcts	Areas of tissue death due to lack of oxygen. Common in stroke patients. Multi-infarct dementia can cause a dementia similar to AD. Infarcts/microinfarcts could also be a hemodynamic contributor to ALS.
Ventricular dilation	Enlargement of the ventricles may occur for a number of reasons, such as loss of brain volume or impaired outflow or absorption of CSF. It has been noted in ALS mice as well as some clinical ALS patients [56, 57].
Degeneration of corticospinal tract(s)	Corticospinal tracts carry nerve impulses from the brain to the spinal cord, with the majority of fibers crossing at the medulla. Involved in voluntary movement. Both anterior and lateral corticospinal tract degeneration is common in ALS [58].
Denervation	Loss of nerve supply to a given region. Retrograde retraction from the neuromuscular junction is thought to initiate denervation in ALS [59, 60].
Neuronal loss	Neurodegenerative diseases such as ALS are characterized by the loss of certain neurons. ALS, specifically, is characterized by the loss of neurons in regions such as the spinal cord, brainstem, and other areas of the brain, especially the motor cortex [3].
Betz cell (loss)	Large neurons localized to the primary motor cortex. Loss of Betz cells is a feature of motor neuron disease [61].
Purkinje cell (loss)	Large neurons localized to the cerebellum. Loss of Purkinje cells has been implied in ALS. One theory suggests that abnormal trafficking and proteolytic processing of the P2X(4) receptor protein may be involved [29].
Pigmentary incontinence	Caused by external deposits of (typically) intracellular pigments. Is as an indicator of the loss of neurons in pigmented nuclei [62, 63]. We specifically assess the locus ceruleus and substantia nigra, for which pigmentary incontinence has been associated with PD.
Neural atrophy	Shrinking of the neural structure, and more specifically of the ventral roots, diaphragm, paraspinal muscle nerve fiber, and general brain and spinal cord, which have been shown in ALS [17].
AD	Neurodegenerative disorder that typically causes dementia. The most common pathological biomarker is amyloid β plaques or tangles. AD in this study was diagnosed based on pathological CERAD criteria [18, 22].
FTLD	A pathological process that occurs in FTD. Characterized by atrophy in the frontal and temporal lobe of the brain, with sparing of the parietal and occipital lobes. Seen in combination with ALS in some patients [55].

tween the onset of ALS symptoms and patient death), and prescribed riluzole usage. These parameters were obtained from the Emory University Electronic Medical Records Database.

Neuropathology Parameters

Table 1 enumerates and describes the significance of the microscopic and macroscopic pathological features examined in this study. Broadly categorized, they include several different cellular pathological markers; circulatory findings; neural atrophy by location; neuronal loss by specific location or by cell type; presence of NFTs by specific location, and diagnoses of other possible disease in conjunction with ALS, including AD based on pathological criteria of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [18] and FTLT based on the postmortem presence of tau, ubiquitin, or FUS-positive inclusions.

Subgroup Definitions

Given previously published reports on the impact of onset age [19], suspected AD [20], ALS onset type [21], and the presence of TDP-43 inclusions [11] on the ALS neuropathology, we divided the overall population into these aforementioned subgroups to examine possible differences in their pathological and clinical features.

- Suspected pathological AD: diagnosis was strictly based on pathological published CERAD criteria [18, 22]. Given the 10-year timeline of the autopsies, the CERAD criteria were utilized instead of recent criteria [e.g. 23] to maintain uniformity throughout the study duration.
- ALS onset type: based on recorded clinic survey response of the first symptom location and type, patients were categorized as 'limb' (i.e. first symptom appears in muscles of an extremity) or 'bulbar' onset (i.e. first symptom appears in the facial muscles or muscles of the throat or tongue).
- Onset age: two separate subgroups of patients were utilized: (1) patients with an onset age of less than 50 years and (2) patients with an onset age greater than 60 years.
- Riluzole treatment: patients undergoing continuous treatment with riluzole for the disease duration (from diagnosis through death) based on clinic medical records.
- Severe TDP-43 immunohistochemistry findings: severe TDP-43 classification was based on the extent of staining and corresponding parametric neuropathological feature degree of severity ranking of 4–6 ('moderate') or 7–9 ('severe') described in Analysis.

Analysis

Given the size of the study population, the goal of the analysis was to find potentially interesting or compelling relationships for further research, as opposed to striving for statistical significance. Three different assessments were utilized for each measure: pathological feature prevalence; pathological feature severity, and assessment of relationships between measured pathological features and clinical parameters (age, gender, onset, etc.).

Pathological Feature Prevalence. Pathological feature prevalence is calculated by determining the percentage of patients with a positive finding independent of severity.

Pathological Feature Severity. Pathological feature degree of severity is calculated using the mode of the neuropathologist's severity rating. A parametric severity scale ranging from 1 to 9 was utilized to quantify the severity of each positive pathological feature

(e.g. spinal cord atrophy), with 1 equating to a sparse within-patient presence and 9 representing the most severe presence. Qualitatively, this scale translates to the following relative rankings: 1–3 = mild (e.g. sparse or minimal intensity); 4–6 = moderate (e.g. marked, pronounced, or persistent intensity); 7–9 = severe (e.g. confluent or ubiquitous intensity). The statistical mode (i.e. rating seen the most frequently within a population) of the parametric severity was utilized to assess pathological feature degree of severity.

Pathological Feature Relationships. Relationships between measures are calculated using a cross-correlational analysis of parameterized pathological measures and temporal clinical parameters (i.e. onset age, age at death, etc.). Cross-correlation analysis was performed utilizing MathWorks MATLAB software to assess possible relationships using previously published relational analysis methods [24, 25]. A cross-correlation greater than 0.3 was conservatively considered a potentially 'promising' relationship worthy of possible further study.

Clinical Relationships. Potential clinical relationships between gender, onset age, and disease duration were also independently assessed for the subgroups shown in table 2 using a standard Student's t test with $p < 0.05$ signifying possible significance. To insure conservative calculations while using multiple t test comparisons, standard Bonferroni corrections were applied to the presented results. Normality of the data distributions was determined using the Shapiro-Wilk test. Alternate confirmatory analysis was also performed using the Mann-Whitney test.

Results

The patient population consisted of 46 subjects, including 29 males and 17 females with an overall onset age of 55.8 years with a standard deviation of 12.7 years. Based on the subgroups described in the Methods, of the 46 patients, 10 (22%) met the CERAD criteria for pathological AD [18]; 13 patients (28%) had an onset age less than 50 years; 18 patients (39%) had an onset age greater than 60 years; 30 patients (65%) had a confirmed limb onset; 12 patients (26%) had a confirmed bulbar onset, and 29 patients (63%) received continuous riluzole treatment.

Gender, Onset and Disease Duration

We assessed the possible role of gender differences, ALS onset age, and disease duration. Table 2 shows for the overall population, each gender, and for each subgroup, the average onset age and standard deviation and average disease duration and standard deviation. Assessment of gender differences found no statistically significant difference between the onset age or disease duration of males and females within the total population or within the assessed subgroups as noted in table 2A. Comparison of onset age among the different subgroups found the

Table 2. Impact of gender, onset, and disease duration**A** Gender, onset age and disease duration for the total study population and for each subgroup

Subgroup	All			Male			Female		
	n	onset age	disease duration	n	onset age	disease duration	n	onset age	disease duration
All	46	55.8±12.7	4.1±2.8	29	55.7±14	4.4±3.1	17	56.1±10.6	3.8±2.1
AD	10	63.6±8.4	3.9±2.8	6	67.2±6.6	3.3±2.3	4	58.3±8.7	4.8±2.1
Non-AD	36	53.7±13	4.2±2.9	23	52.7±14	4.7±3.3	13	55.5±11.3	3.4±2.1
<50 years	13	40.4±9.3	5.8±3.6	9	39.3±10.7	6.8±3.6	4	43±5.1	3.7±2.8
>60 years	18	68.1±4.6	2.6±1.4	12	68.2±4.8	2.3±1.1	6	68±4.5	3.3±1.8
Bulbar	12	62.3±13	3.6±3.4	9	61.8±14.1	2.9±3.6	3	63.9±11.7	5.7±1.4
Limb	30	53.3±10.5	4.3±2.9	18	52.6±12	5.2±3.2	12	54.4±8.4	3.1±1.7
Riluzole	29	55.9±11	3.6±2	18	56.2±12.3	3.3±2.1	11	55.3±9.3	3.9±1.9
Non-riluzole	8	58.1±10.3	4.7±3.6	4	57.1±11.1	6.8±3.8	4	59.1±10.9	2.6±2
Severe TDP-43	26	57.9±10.8	4.1±2.3	17	58.2±10.6	3.9±2.3	9	57.2±11.7	4.5±2.3

No statistically significant difference in onset or disease duration was found between males and females within any of the subgroups. Onset age and disease duration are shown as an average in years ± the standard deviation.

B Assessment for statistical significance of gender, onset, and disease duration in the subgroups

Comparison	All onset	All duration	Male onset	Male duration	Female onset	Female duration
All to AD	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
AD to non-AD	<0.05*	>0.05	<0.05*	>0.05	>0.05	>0.05
Onset <50 to >60 years	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	>0.05
Bulbar to limb	<0.05*	>0.05	>0.05	>0.05	>0.05	>0.05
Rilutek to non-rilutek	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
All to severe TDP-43	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

* $p < 0.05$; ** $p < 0.001$.

following statistically significant differences, as denoted in table 2B: comparison of the AD versus non-AD subgroups showed that the onset is later in suspected male AD patients ($p = 0.03$); comparison of the bulbar versus the limb onset showed that onset is later in bulbar onset patients ($p = 0.02$); comparison of disease duration between the different subgroups found that the male older onset patients (>60 years of age at onset) had a shorter disease duration compared to the male young onset patients (<50 years of age at onset). There were no statistically significant differences in onset age or disease duration for the riluzole and severe TDP-43 subgroups.

Population Prevalence of Pathological Features

We began our pathological assessment by simply determining the prevalence of each pathological feature in each ALS patient subgroup. The prevalence assessment

only takes into account the percentage of patients having a positive finding but does not take into account the feature degree of severity, which is assessed separately. Table 3 lists the prevalence of pathological cellular markers, circulatory measures, neuronal loss by location, NFTs by location, neural atrophy by location, and pathological dementia for the total ALS population and for each subgroup.

Feature Prevalence in the Total Population. Of the pathological markers, TDP-43 was the most prevalent with 80% of the total 46-patient population showing positive inclusions, followed by amyloid β (35%), tauopathy (17%) and Lewy body formation (11%). Atherosclerosis was the most common feature of the four circulatory measures with an overall prevalence of 35%. The prevalence of anterior and lateral corticospinal tract degeneration and denervation was high, ranging from 67 to 89%;

Table 3. Prevalence of a positive finding for each pathological feature for the total ALS study population as well as for the subgroups of AD, non-AD, <50 years of age at onset (<50 years), >60 years at onset (>60 years), continuous riluzole treatment (riluzole), those who never took riluzole (non-riluzole), and those with severe TDP-43 findings

Assessment	All		AD		Non-AD		<50 years		>60 years		Limb onset		Bulbar onset		Riluzole		Non-riluzole		Severe TDP-43		
	Sample:	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n
	Size:	100	46	22	10	78	36	28	13	39	18	65	30	26	12	63	29	17	8	57	26
Pathological markers																					
α-Synuclein		4	2	0	0	6	2	0	0	6	1	3	1	0	0	3	1	0	0	4	1
β-Amyloid		35	16	80	8	22	8	8	1	44	8	33	10	50	6	41	12	50	4	38	10
Lewy body formation		11	5	10	1	11	4	8	1	17	3	13	4	8	1	7	2	13	1	4	1
Tauopathy		17	8	10	1	19	7	23	3	6	1	20	6	17	2	21	6	0	0	15	4
TDP-43		80	37	80	8	81	29	85	11	78	14	77	23	75	9	83	24	63	5	100	26
Circulatory																					
Atherosclerosis		35	16	60	6	28	10	8	1	44	8	30	9	33	4	38	11	13	1	38	10
Infarcts		2	1	0	0	3	1	0	0	6	1	0	0	8	1	0	0	13	1	4	1
Microinfarcts		7	3	0	0	8	3	0	0	17	3	3	1	17	2	7	2	13	1	12	3
Ventricular dilation		13	6	30	3	8	3	0	0	28	5	10	3	17	2	14	4	0	0	23	6
Tract degeneration and denervation																					
Anterior corticospinal tract		72	33	80	8	69	25	69	9	61	11	73	22	50	6	66	19	75	6	81	21
Lateral corticospinal tract		89	41	100	10	86	31	77	10	89	16	87	26	92	11	86	25	88	7	92	24
Denervation		67	31	80	8	64	23	69	9	61	11	73	22	67	8	69	20	63	5	69	18
Neuronal loss																					
All (any area)		83	38	80	8	83	30	69	9	83	15	87	26	75	9	83	24	100	8	85	22
Amygdala		4	2	0	0	6	2	8	1	6	1	3	1	0	0	3	1	0	0	8	2
Anterior horns		43	20	30	3	47	17	23	3	50	9	43	13	58	7	45	13	50	4	46	12
Betz cells		76	35	80	8	75	27	69	9	78	14	73	22	83	10	72	21	75	6	81	21
Dentate nucleus		17	8	20	2	17	6	23	3	6	1	17	5	8	1	17	5	13	1	19	5
Gyrus		2	1	0	0	3	1	0	0	6	1	3	1	0	0	3	1	0	0	4	1
Locus ceruleus		13	6	20	2	11	4	15	2	11	2	13	4	0	0	10	3	13	1	8	2
Medulla		33	15	40	4	31	11	23	3	28	5	30	9	33	4	34	10	38	3	35	9
Purkinje cells		85	39	90	9	83	30	85	11	83	15	83	25	75	9	86	25	75	6	100	26
Spinal cord		24	11	40	4	19	7	31	4	17	3	23	7	25	3	24	7	38	3	31	8
Substantia nigra		13	6	20	2	11	4	8	1	11	2	10	3	17	2	10	3	0	0	8	2
Neural atrophy																					
Brain		20	9	30	3	17	6	8	1	28	5	13	4	33	4	17	5	38	3	31	8
Diaphragm		54	25	60	6	53	19	54	7	50	9	63	19	58	7	59	17	50	4	54	14
Paraspinal muscle fiber		57	26	60	6	56	20	62	8	50	9	60	18	58	7	59	17	38	3	58	15
Spinal cord		9	4	0	0	11	4	15	2	6	1	3	1	17	2	10	3	0	0	4	1
Spinal ventral nerve roots		65	30	90	9	58	21	54	7	61	11	67	20	67	8	69	20	63	5	81	21
Pigmentary incontinence																					
Locus ceruleus		76	35	80	8	75	27	69	9	78	14	73	22	83	10	83	24	63	5	85	22
Substantia nigra		70	32	70	7	69	25	62	8	67	12	70	21	83	10	72	21	63	5	73	19
NFTs																					
All (any area)		78	36	100	10	72	26	62	8	83	15	73	22	83	10	83	24	63	5	88	23
Amygdala		43	20	80	8	33	12	23	3	50	9	43	13	50	6	52	15	50	4	38	10
Anterior horn cells		4	2	10	1	3	1	8	1	0	0	7	2	0	0	7	2	0	0	0	0
Basal ganglia		2	1	0	0	3	1	8	1	0	0	0	0	8	1	3	1	0	0	0	0
Caudate nucleus		2	1	10	1	0	0	0	0	6	1	0	0	8	1	3	1	0	0	4	1
Cingulate cortex		4	2	0	0	6	2	0	0	6	1	0	0	0	0	0	0	0	0	8	2
Dentate nucleus		2	1	0	0	3	1	0	0	0	0	3	1	0	0	0	0	13	1	4	1
Entorhinal cortex		61	28	80	8	56	20	31	4	72	13	57	17	58	7	66	19	63	5	69	18
Frontal cortex		13	6	10	1	14	5	23	3	11	2	17	5	17	2	17	5	0	0	19	5
Hippocampus		37	17	80	8	25	9	23	3	50	9	27	8	58	7	41	12	38	3	38	10

Table 3 (continued)

Assessment	All		AD		Non-AD		<50 years		>60 years		Limb onset		Bulbar onset		Riluzole		Non-riluzole		Severe TDP-43			
	Sample:		%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n		
	Size:		100	46	22	10	78	36	28	13	39	18	65	30	26	12	63	29	17	8	57	26
Hypothalamus			22	10	30	3	19	7	8	1	28	5	17	5	25	3	17	5	38	3	27	7
Insular cortex			22	10	50	5	14	5	8	1	33	6	20	6	25	3	21	6	13	1	27	7
Locus ceruleus			30	14	40	4	28	10	23	3	22	4	27	8	25	3	28	8	38	3	38	10
Neocortex			4	2	0	0	6	2	8	1	0	0	3	1	8	1	7	2	0	0	0	0
Nucleus basalis			43	20	40	4	44	16	31	4	50	9	30	9	67	8	34	10	50	4	62	16
Occipital cortex			2	1	0	0	3	1	0	0	0	0	3	1	0	0	3	1	0	0	0	0
Parietal cortex			2	1	10	1	0	0	0	0	0	0	3	1	0	0	3	1	0	0	0	0
Putamen			2	1	10	1	0	0	0	0	6	1	0	0	8	1	3	1	0	0	4	1
Substantia nigra			13	6	20	2	11	4	15	2	17	3	10	3	17	2	14	4	13	1	15	4
Temporal cortex			15	7	10	1	17	6	8	1	22	4	13	4	17	2	17	5	13	1	23	6
Thalamus			15	7	30	3	11	4	8	1	28	5	10	3	25	3	21	6	0	0	19	5
Dementia																						
AD (pathological)			22	10	100	10	0	0	0	0	33	6	20	6	25	3	31	9	0	0	15	4
FTLD (pathological)			26	12	30	3	25	9	0	0	33	6	17	5	42	5	24	7	38	3	42	11

pigmentary incontinence also had a similar prevalence. Neural atrophy was most prevalent in the spinal ventral roots (65%) and paraspinal muscle fiber (57%). Of the total population, 83% of the patients had a positive finding for neuronal loss in one or more of the 10 assessed locations (table 3). The cells or locations with the highest prevalence included Purkinje cells (85%), Betz cells (76%), anterior horn of the spinal cord (43%), and the medulla (33%). NFTs were present in one or more of the 20 assessed locations in 78% of the total ALS population. NFT prevalence was the highest in the entorhinal cortex (61%), amygdala (43%), nucleus basalis (43%), hippocampus (37%), hypothalamus (22%) and insular cortex (22%).

Feature Prevalence in the AD versus Non-AD ALS Subgroup. Not surprisingly, the AD subgroup had a substantially higher prevalence of amyloid β (80%) compared to the non-AD group (22%). Atherosclerosis and ventricular dilation were also more prevalent in the AD subgroup, but this finding appears to correlate more with age than it does with the presence of AD (i.e. the AD patients had an overall later ALS onset). NFTs were also substantially more prevalent in the AD subgroup, with NFTs present in 100% of the AD patients. More specifically, the prevalence of NFTs was a factor of 2 higher in the amygdala and hippocampus compared to the non-AD subgroup. Even with the application of an age-matching correction, the prevalence of amygdala NFTs remains a factor of 1.75

times greater in the AD population, which is indicative of an AD-specific relationship.

Feature Prevalence in the <50-Year versus >60-Year Onset Age Subgroup. Most notably, the <50-year onset age subgroup had negligible circulatory findings while the >60-year onset age subgroup had qualitatively above average prevalence. Most notable in the >60-year onset age group was the prevalence of atherosclerosis (44%) and ventricular dilation (28%). Additionally, the <50-year onset age subgroup had a lower prevalence of NFTs both overall as well as in specific locations. More specifically, NFTs were a factor of 2 greater in the amygdala, a factor of 3 greater in the entorhinal cortex, and a factor of 4 greater in the insular cortex in the >60-year onset age subgroup.

Feature Prevalence in the Bulbar versus Limb Onset Subgroups. Pathological markers, tract degeneration, neuronal loss, neural atrophy, and pigmentary incontinence were all very similar between limb and bulbar onset subgroups. While the overall presence of NFTs was also similar, there were a few potentially notable differences in the prevalence of NFTs at specific locations. For example, the prevalence of NFTs in the hippocampus and nucleus basalis and the prevalence of FTLD was a factor of 2 more prevalent in the bulbar onset subgroup.

Feature Prevalence in the Riluzole versus Non-Riluzole Subgroups. The non-riluzole subgroup had a small sample size (8 patients). Given the sample size, there are no substantial differences noted in feature prevalence be-

tween the riluzole and non-riluzole subgroups. Nonetheless, it is of potential interest that 100% of the non-riluzole patients had neuronal loss in one or more locations compared to 83% in the riluzole group and 85% in the total population.

Feature Prevalence in the Severe TDP-43 Subgroup. The TDP-43 subgroup was compared to the total population. Interestingly 100% (26 patients) of the severe TDP-43 subgroup were found to have some form of Purkinje cell neuronal loss as assessed using the modified Bielschowsky silver method. Also potentially notable was that spinal nerve atrophy was a factor of 1.3 greater and FTLD was a factor of 1.5 greater in the severe TDP-43 subgroup. No notable differences were denoted in the prevalence of overall NFTs or localized NFTs.

Severity of Pathological Features

Next, we examined the severity of the pathological features independent of prevalence. For example, are there some pathological features that are not prevalent (e.g. few patients have a positive finding) but when present, the degree of severity is typically 'severe'? In contrast, are there features that are very prevalent (e.g. many patients have a positive finding), but the degree of severity is typically 'mild'?

Feature Severity in the Total ALS Population. Of the patients with circulatory findings, the severity was mild. Neuronal losses varied from mild to moderate. Moderate neuronal losses were present in the overall spinal cord, anterior horns of the spinal cord, and the dentate nucleus, while the remaining areas shown in table 3 had only mild losses. Interestingly, some of the areas of neuronal loss with the greatest prevalence in the patient population had mild severity, including Purkinje cells, Betz cells, and neuronal losses in the medulla. NFT severity varied the most by location, from mild to severe, and was loosely correlated with patient age; interestingly, there was negligible correlation of NFT severity with disease duration. NFTs, when present, were graded as severe in the nucleus basalis, entorhinal cortex, and thalamus and moderate in the locus ceruleus, amygdala, insular cortex, anterior horns, and substantia nigra. Pigmentary incontinence, when present, was graded as mild in both the substantia nigra and locus ceruleus. The degree of severity of brain atrophy and spinal cord atrophy was graded equivalently moderate.

Feature Severity between Subgroups. For the most part, the overall ALS population and the individually assessed subgroups shown in table 3 had equivalent severity ratings for each of the assessed pathological features. How-

ever, there were a couple of exceptions in the AD subgroup. More specifically, the NFTs in the amygdala of the AD subgroup were very severe compared to the mild to moderate severities seen in the other subgroups. Additionally, paraspinal muscle atrophy and Betz cell loss were moderate in the AD subgroup, whereas they were only mild in the other subgroups. Another substantial difference in the severity of subgroup pathological features was with onset age. The <50-year onset age subgroup only had mild to occasionally moderate NFTs with no single location rated at severe; in contrast, the degree of severity of NFTs was typically graded as severe in the >60-year onset age subgroup.

Relationships between Pathological Measures or Patient Subgroups

As denoted in the Methods, due to the large number of assessed measures and the lesser number of patients in the population and/or subgroups, statistical analysis was limited to the identification of 'promising' relationships rather than traditional statistical significance. Relational and cross-correlation analysis takes into account both prevalence (number of patients with a positive finding) and severity (quantitative degree of positive finding based on the neuropathologist's rating) to identify possible relationships between two parameters. As sanity checks, we first examined the cross-correlations between known relationships (e.g. cross-correlation between onset age and age at death, $r = 0.98$; paraspinal muscle atrophy and diaphragm atrophy, $r = 0.84$, etc.).

Relationship Assessment in the Total Population. We examined the cross-correlation between each clinical and pathological measure, 2,279 possible relationships in total (see online supplementary table 1, www.karger.com/doi/10.1159/000433581). Key findings connected areas of neuronal loss and NFTs by functional connections in the brain; for example, the hippocampus, amygdala, and entorhinal cortex all had strong relationships for neuron loss and NFTs ($r = 0.5$ – 0.8). Also not unexpected is that circulatory measures were loosely correlated with age, with findings in the range of $r = 0.3$ – 0.4 . TDP-43 inclusions were correlated with Purkinje cell loss ($r = 0.52$) and FTLD ($r = 0.46$). Brain atrophy was most correlated with amygdala NFTs as well as FTLD ($r = 0.47$). However, neuronal losses in the brain were most correlated with loss of neurons in the dentate nucleus and medulla ($r > 0.5$) and to a slightly lesser degree, neuronal losses in the spinal cord, locus ceruleus, and Purkinje cells ($r > 0.4$).

Assessment of Subgroup-Specific Pathological Relationships. There were a few substantial subgroup-specific pathological relationships. Compared to limb onset, bulbar onset ALS correlated more strongly with anterior and lateral corticospinal tract degeneration as well as patient age at death. Further assessment of upper limb onset versus lower limb onset revealed no notable relationship to any of the examined clinical or pathological parameters. An examination of the relationships with pathological AD did find an expected moderate relationship between amyloid β immunohistochemistry ($r = 0.5$) and hippocampus and amygdala NFTs ($r = 0.56$). Interestingly, despite the fact that 80% of the overall ALS population had a positive finding for TDP-43 inclusions, there was no notable clinical or pathological relationship difference between those with 'severe' TDP-43 inclusions versus 'mild' inclusions. Finally, there were no substantial pathological relationships in the cross-correlation analysis that contained gender as a parameter.

Discussion

We assessed the prevalence, severity, and possible relationships between numerous clinical parameters and pathological features from the autopsies of 46 patients with sporadic ALS. The most prevalent and severe pathological features encompassed TDP-43 inclusions, NFTs, neuronal losses, and corticospinal tract degeneration – all of which were individually identified in greater than 75% of the total ALS study with individual feature degree of severity ratings ranging from moderate to severe. Additionally, our overall results found only minimal impacts of gender, continuous riluzole treatment, onset type, and severe TDP-43 pathology, on discerning differences between these subgroups and the overall ALS patient study population. However, notable differences were identified in the AD subgroup, less than 50-year onset age subgroup, and greater than 60-year onset age subgroups.

Onset, Gender, and Disease Duration

Our finding that a later ALS disease onset is associated with bulbar onset patients and with patients that appear to have comorbid AD is consistent with prior work [21]. Also, as has been previously shown, we found that gender does not have a clear impact on clinical patient survival [19], despite reports that female transgenic SOD1 mice tend to have a longer disease duration [e.g. 26, 27]. Further study in larger patient populations is necessary to establish the potential role of gender on clinical ALS parameters.

TDP-43

While other studies have also found that TDP-43 inclusions were 'common' in sporadic ALS, even in those patients without actual TDP-43 mutations [11, 12], this is one of the first studies to quantitatively approximate that more than 80% of patients have a positive finding for abnormal TDP-43 inclusions and, of these, the within-patient degree of severity ranges from moderate to very severe.

Betz and Purkinje Cell Loss

Betz cell loss, which is known to be significant in ALS [28], was found to be very prevalent in this population (76%), although the within-patient severity was typically mild. Similarly, we also found that Purkinje cell loss is a prevalent pathological feature, which has previously only been identified in ALS animal models [29]. Nonetheless, while >80% of patients in this study had Purkinje cell losses, the degree of within-patient severity was typically mild.

Atrophy and Degeneration

We also confirm the known correlation between spinal ventral root atrophy and diaphragm atrophy [30] while calculating its pathological prevalence in this population at 65 and 57%, respectively. Finally, we affirm that corticospinal tract degeneration in ALS-FTD is a distinguishing feature that is typically not present in FTD alone [1] and is more strongly correlated with bulbar onset ALS compared to limb onset [31].

Neurofibrillary Tangles

There were several interesting findings regarding NFTs. The finding that ALS patients meeting postmortem pathological AD diagnostic criteria tend to have a higher prevalence and severity of NFTs, especially in the amygdala, hippocampus, entorhinal cortex, and insular cortex, was not necessarily unexpected [32]. However, the 80% prevalence of NFTs identified in the overall ALS study population was notable; moreover, the within-patient degree of NFT severity was typically moderate to severe. Previously, NFTs have mostly been associated with other comorbid disease such as AD [33], PD [34], Pick's disease [35] and other pathologies that include FTD or FTLD [36]. Like the aforementioned study [34], we also found that NFTs were less prevalent in the <50-year onset age subgroup, and the within-patient degree of severity of NFTs loosely correlated with patient age at death. However, unlike AD-only patients [37], neuronal losses and NFTs did not strongly correlate in ALS patients

($r = 0.22$). Of all the assessed pathological features, it is the entorhinal cortex NFTs which have the strongest correlation with ALS clinical disease duration ($r = 0.32$). NFTs in the entorhinal cortex and hippocampus have previously been associated with Guamanian ALS-PD disorder [34]. While entorhinal cortex NFTs were pervasive in the overall ALS population (61% prevalence, 'severe' degree rating), hippocampus NFTs were less so (37% prevalence, 'moderate' degree rating). In contrast, entorhinal cortex NFTs and hippocampus NFTs were both simultaneously prevalent and severe in the AD subgroup of ALS patients (80% prevalence, 'severe' degree rating).

Based on the overall pathological findings of this study for cellular pathological markers, atrophy, neuronal losses, and NFTs by location, we find that three major functional areas are affected in ALS: (1) motor control (entorhinal cortex, Betz cell loss, Purkinje cell loss, corticospinal tract degeneration, spinal atrophy, ventral root atrophy, etc.); (2) memory, cognition, and behavior (hippocampus, amygdala, β -amyloid, etc.); and (3) autonomic and homeostatic function (medulla, insular cortex and hypothalamus). Motor control and memory dysfunction is not really a surprising finding. However, autonomic and homeostatic control (including basic body operation, like blood pressure) is less discussed in pathological studies. Nonetheless, autonomic function [7, 8], blood pressure regulation [38–41], and even the potential role of antecedent disease [42] and its effect on homeostasis [42, 64], have been previously discussed in the literature. In

summary, the widespread identification of overlapping pathological features quantified in this study lends credence to the hypothesis that ALS, FTD, as well as other neuropathologies could potentially share interrelating etiologies [1, 2, 9], which may represent a larger motor-neurodegenerative-cognitive neuropathological continuum. Clearly, more research is needed to differentiate between disease-specific and shared neuropathological features.

Limitations and Future Directions

As noted in the Methods, given the time span of the study, not all genetic tests were available for all patients. It is possible that more recently identified mutations, such as repeat expansions in the C9ORF72 gene [43], could impact the subgroup results. Finally, future similar studies would benefit from newly developed quantitative methods for assessing TDP-43, NFTs, FTL, and other key markers, which were not available at the initiation of the present study [23, 43–46].

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