

# Multimodal longitudinal study of structural brain involvement in amyotrophic lateral sclerosis

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## Abstract

### Objective

To understand the progressive nature of amyotrophic lateral sclerosis (ALS) by investigating differential brain patterns of gray and white matter involvement in clinically or genetically defined subgroups of patients using cross-sectional, longitudinal, and multimodal MRI.

### Methods

We assessed cortical thickness, subcortical volumes, and white matter connectivity from T1-weighted and diffusion-weighted MRI in 292 patients with ALS (follow-up: n = 150) and 156 controls (follow-up: n = 72). Linear mixed-effects models were used to assess changes in structural brain measurements over time in patients compared to controls.

### Results

Patients with a *C9orf72* mutation (n = 24) showed widespread gray and white matter involvement at baseline, and extensive loss of white matter integrity in the connectome over time. In *C9orf72*-negative patients, we detected cortical thinning of motor and frontotemporal regions, and loss of white matter integrity of connections linked to the motor cortex. Patients with spinal onset displayed widespread white matter involvement at baseline and gray matter atrophy over time, whereas patients with bulbar onset started out with prominent gray matter involvement. Patients with unaffected cognition or behavior displayed predominantly motor system involvement, while widespread cerebral changes, including frontotemporal regions with progressive white matter involvement over time, were associated with impaired behavior or cognition. Progressive loss of gray and white matter integrity typically occurred in patients with shorter disease durations (<13 months), independent of progression rate.

### Conclusions

Heterogeneity of phenotype and *C9orf72* genotype relates to distinct patterns of cerebral degeneration. We demonstrate that imaging studies have the potential to monitor disease progression and early intervention may be required to limit cerebral degeneration.

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## Glossary

**ALS** = amyotrophic lateral sclerosis; **ALS-FTD-Q** = Amyotrophic Lateral Sclerosis–Frontotemporal Dementia Questionnaire; **ALSFRS-R** = ALS Functional Rating Scale–revised; **DWI** = diffusion-weighted imaging; **ECAS** = Edinburgh Cognitive and Behavioral ALS Screen; **FAB** = frontal assessment battery; **FDR** = false discovery rate; **FA** = fractional anisotropy; **LME** = linear mixed-effects; **PAN** = Prospective ALS Study The Netherlands; **VFI** = verbal fluency index.

Amyotrophic lateral sclerosis (ALS) is clinically and genetically a heterogeneous disease.<sup>1</sup> Up to 15% of patients develop frontotemporal dementia, and another 35% have some degree of cognitive or behavioral impairment.<sup>2,3</sup> A repeat expansion in *C9orf72* appears to be the most common mutation in patients with ALS.<sup>4,5</sup> The pathologic mechanisms underlying the process of neurodegeneration in ALS have not yet been resolved. Knowing more about how neurodegeneration develops over time and about its associated biomarkers in patients with ALS and subgroups, based on phenotype or genotype, may help to identify therapy that slows or stops disease progression.

Neuroimaging might be valuable in tracing disease progression in ALS. Previous cross-sectional imaging studies have consistently reported abnormalities within the motor area,<sup>6</sup> frontotemporal regions,<sup>7,8</sup> and basal ganglia.<sup>9,10</sup> It has also become clear that patients with a *C9orf72* repeat expansion have a distinct neuroimaging phenotype of widespread gray and white matter involvement.<sup>11,12</sup> Longitudinal imaging studies may provide an anatomical characterization of disease progression related to clinical phenotype, and might guide our understanding of underlying neurodegenerative processes. Moreover, whereas many imaging studies applied a single imaging technique, a multimodal approach will be valuable for establishing the relative sensitivity of various imaging techniques in different stages of ALS and thus provide greater insight into when gray and white matter become involved during disease development.

In the present study, we investigated longitudinal structural brain changes in a large cohort of patients with ALS, applying a multimodal imaging approach, assessing both gray and white matter of the brain.

## Methods

### Participants

Patients were recruited from Prospective ALS Study The Netherlands (PAN), an ongoing population-based, case-control study performed in the Netherlands since January 2006, shortly after diagnosis.<sup>13</sup> Clinical characteristics, including functional, cognitive, and behavioral status, disease duration, and site of onset, were recorded. Functional status was assessed using the revised ALS Functional Rating Scale–revised (ALSFRS-R).<sup>14</sup> Cognition was assessed using 3 neuropsychological tests: verbal fluency index (VFI), Edinburgh Cognitive and Behavioral ALS Screen (ECAS), and

frontal assessment battery (FAB). Abnormality in at least one of these tests was regarded as cognitive impairment. Because ECAS was developed after the study started, different methodologies to assess cognitive or behavioral impairment were applied. VFI scores were transformed to *z* scores. A patient had abnormal cognition if the score was below the 5th percentile.<sup>15</sup> The ALS-specific scores of the ECAS<sup>16,17</sup> were used to assess cognitive status: a patient was labeled with abnormal cognition if the residual score (i.e., observed score – expected score) was in the lowest 5% of the Dutch normative population, derived using the percentile rank method on residuals.<sup>16</sup> FAB scores <13 points indicated abnormal cognition.<sup>18</sup> Behavior was assessed using the Amyotrophic Lateral Sclerosis–Frontotemporal Dementia Questionnaire (ALS-FTD-Q)<sup>19</sup> and the behavioral screen of the ECAS (asking the caregiver to report changes in behavior).<sup>17</sup> Behavioral impairment was defined as apathy or scoring at least 2 other items on the ECAS behavioral screen<sup>3</sup> or an ALS-FTD-Q score  $\geq 22$  points.<sup>19</sup>

Disease duration in months was calculated from symptom onset to scan date. Disease progression rate was calculated by  $(48 - \text{ALSFRS-R score})/\text{disease duration (in months)}$ .<sup>20</sup> *C9orf72*, *FUS*, *TARDBP*, and *SOD1* mutations were assessed, as described previously.<sup>21</sup> Follow-up of patients was conducted approximately every 4–6 months. At each visit, patients were examined by a clinician specialized in motor neuron diseases, and their functional, cognitive, and behavioral status assessed.

Controls also came from the aforementioned PAN study.<sup>13</sup> Follow-up scans were conducted for this reference group after approximately 1 year to account for physiologic changes over time (“aging”). Patients with a history of epilepsy, stroke, or any overt structural brain abnormalities were excluded.

### Image acquisition and preprocessing

Anatomical T1- and diffusion-weighted images of the brain were acquired using a 3T Philips (Best, the Netherlands) Achieva Medical Scanner, as described previously.<sup>22,23</sup>

Cortical and subcortical brain regions were analyzed using T1 images and FreeSurfer (V.5.3.0, [surfer.nmr.mgh.harvard.edu/](http://surfer.nmr.mgh.harvard.edu/)). In total, 92 distinct brain regions (68 cortical, 14 subcortical, and 6 ventricular regions, 4 cerebellar volumes) were parcellated and segmented (editing cortical parcellations was not necessary)<sup>24</sup> according to the Desikan-Killiany atlas.<sup>25</sup> Longitudinal analysis was performed by creating an unbiased within-subject template space and image using robust

inverse consistent registration.<sup>26</sup> Participants with a single scan were also preprocessed within the longitudinal preprocessing stream of FreeSurfer.

Using diffusion-weighted imaging (DWI), white matter tracts forming the structural brain network were reconstructed based on fractional anisotropy (FA).<sup>22,23</sup> Nodes were taken as 83 segmented brain regions (cortical and subcortical regions and brainstem) and the interlinking connections represented the white matter pathways. For each participant, an individual undirected, weighted brain network was reconstructed.<sup>22</sup> To avoid potential spurious fibers, we only considered connections that were present in 50% of all participants.<sup>27</sup>

## Statistical analyses

Statistical analyses were performed using the R software package version 3.4.3 (R Foundation for Statistical Computing, R-project.org). Connectome-based analyses were implemented using MATLAB Release 2017b (The MathWorks, Inc., Natick, MA; mathworks.com). Measures of cortical thickness, subcortical volumes, and structural connectivity were statistically compared between patients with ALS and controls. Furthermore, we investigated patients with *C9orf72* separately, as they have a distinct imaging phenotype, and performed sensitivity analyses to examine possible influence of other pathogenic mutations. Age and sex were used as covariates in all analyses. For subcortical volumes, we also included total intracranial volume as covariate.

Cross-sectional analyses were conducted on the baseline scans using linear models and permutation tests (10,000 random permutations). Longitudinal analyses were performed, based on all available scans, applying linear mixed-effects (LME) models to assess change of structural measures over time, while accounting for random between-subject variation using random slopes and random intercepts.<sup>28</sup> Participants with single assessments were included to take into account potential attrition bias and different time intervals between scans were incorporated. LME models are well-suited to analyzing longitudinal data with irregular and different follow-up intervals, as was the case for patients and controls.<sup>28,29</sup> To investigate disease-specific effects in patients with ALS compared to controls, an interaction term (follow-up interval  $\times$  group) was included. Here, follow-up interval denoted the time from baseline scan up to the moment of the follow-up scan and group refers to patient or control. Stratified analyses for site of onset (bulbar vs spinal), cognitive or behavioral impairment, and disease duration were performed to investigate brain involvement in relation to clinical phenotype.

All gray matter analyses were corrected for multiple testing using false discovery rate (FDR). *p* Values  $<0.05$ , after FDR correction, were considered statistically significant. Significance of connectome findings was assessed by means of Network-Based Statistics,<sup>30</sup> using the property of connectome disease effects to be more plausible when occurring in

connected components rather than in isolation. Edges were initially labeled as affected if the linear model indicated a *p* value  $<0.05$ . The size of the largest connected component of these affected connections was tested for significance by comparing it to a null distribution obtained by 1,000 random permutations of group assignments.

Fair comparisons between cognitively or behaviorally impaired and unaffected patients require equal sample sizes. We therefore obtained 50 random subsamples of unaffected patients to equal those of the impaired subgroups, and performed 400 and 40 permutations per subsample for the gray and white matter analyses, respectively, which we subsequently pooled. To achieve result stability and account for subsampling analysis, we carried out twice as many permutations (in total). Sensitivity analyses were performed by excluding all participants with abnormal ECAS scores to assess whether results were driven by participants with cognitive impairment. Finally, we compared baseline scans of patients without follow-up data to those of patients with longitudinal data to assess attrition bias.

## Standard protocol approvals, registrations, and patient consents

The Medical Ethical Committee of the University Medical Center Utrecht approved the study. Written informed consent was obtained from all participants.

## Data availability

The data that support the findings of this study are available via the corresponding author, on reasonable request. The reporting of this study conforms to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement.<sup>31</sup>

# Results

## Demographics

In total, 292 patients with ALS and 156 controls participated in the study (table 1). Twenty-four of the patients tested positive for the *C9orf72* repeat expansion. DWI data were available for 251 patients with ALS (21 *C9orf72*-positive patients) and 135 controls. Follow-up data (up to 6 visits, tables e-1 and e-2, doi:10.5061/dryad.8931zcrkv) were available for 133 *C9orf72*-negative patients with ALS (i.e., patients without the *C9orf72* repeat expansion) and 17 *C9orf72*-positive patients with ALS, with a median follow-up time between the first and second visit of 5.23 months. This relatively short follow-up time was due to the progressive nature of the disease. Genetic data were available for 211 patients and 7 patients had a pathogenic mutation other than the *C9orf72* repeat expansion (table e-3, doi:10.5061/dryad.8931zcrkv). Of the total control group, 72 controls had 2 or 3 follow-up visits (median follow-up time 11.5 months). Participants dropped out at follow-up because they were unable to undergo second scan ( $n = 73$ ),

were deceased at follow-up (n = 58), or second scan was not scheduled yet according to study protocol at time of analysis (n = 102).

To investigate brain involvement in relation to clinical phenotype, *C9orf72*-negative patients were stratified for site of onset (table e-4, doi:10.5061/dryad.8931zcrkv). Patients

were categorized into 4 groups based on quartiles of disease duration at the first MRI scan (i.e., gray matter: ≤9.2, 9.2–13.3, 13.3–21.2, and >21.2 months; white matter: ≤10, 10–14, 14–21, and >21 months; table e-5, doi:10.5061/dryad.8931zcrkv). Cognitive and behavioral status was available for a subset of patients (tables e-6 to e-8, doi:10.5061/dryad.8931zcrkv). Significant correlations between the MRI

**Table 1** Demographic and clinical characteristics of patients with amyotrophic lateral sclerosis (ALS) and controls

	ALS <i>C9orf72</i> -negative		ALS <i>C9orf72</i> -positive		Controls	
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up
<b>Number</b>	268	133	24	17	156	72
<b>Follow-up interval, mo</b>		5.3 (4.2–6.2)		5.1 (3.7–6.4)		11.5 (9.2–17.8)
<b>Male, n (%)</b>	178 (66.4)	89 (66.9)	16 (66.7)	12 (70.6)	102 (65.4)	48 (66.7)
<b>Age at baseline, y</b>	63.4 (54.6–68.5)	61.3 (51.9–66.7)	56.7 (52.1–64.3)	54.3 (51.6–63.1)	61.2 (49.7–66.6)	61.7 (53.9–68.0)
<b>Age at onset, y</b>	61.5 (53.1–66.9)	59.7 (49.8–64.8)	56.1 (50.6–63.3)	53.9 (47.2–61.8)		
<b>Handedness, right, n (%)</b>	224 (83.6)	116 (87.2)	18 (75.0)	14 (82.4)	130 (83.3)	66 (91.7)
<b>Type, familial, n (%)<sup>a</sup></b>	15 (5.6)	9 (6.8)	14 (58.3)	8 (47.1)		
<b>Site of onset, n (%)</b>						
<b>Bulbar</b>	74 (27.6)	30 (22.6)	5 (20.8)	3 (17.6)		
<b>Spinal</b>	192 (71.6)	102 (76.7)	19 (29.2)	14 (82.4)		
<b>Thoracic</b>	2 (0.7)	1 (0.8)				
<b>FTD, n (%)</b>	2 (0.7)		1 (4.2)			
<b>Cognition, n (% abnormal)</b>	208 (16.8)	117 (13.7)				
<b>VFI z scores, n (% abnormal)</b>	144 (11.1)	86 (14.0)				
<b>ECAS ALS specific, n (% abnormal)</b>	118 (9.3)	54 (13.0)				
<b>FAB, n (% abnormal)</b>	182 (8.2)	111 (5.4)				
<b>Behavior, n (% abnormal)</b>	149 (34.2)	84 (29.8)				
<b>ALS-FTD-Q, n (% abnormal)</b>	61 (47.5)	36 (47.2)				
<b>ECAS behavior, n (% abnormal)</b>	83 (34.9)	41 (29.3)				
<b>EI Escorial criteria, n (%)</b>						
<b>Definite</b>	37 (13.8)	16 (12.0)	2 (8.3)	2 (11.8)		
<b>Probable</b>	114 (42.5)	54 (40.6)	5 (20.8)	2 (11.8)		
<b>Probable laboratory-supported</b>	75 (28.0)	39 (29.3)	3 (12.5)	3 (17.6)		
<b>Possible</b>	42 (15.7)	24 (18.1)	14 (58.3)	10 (58.8)		
<b>Progression rate</b>	0.51 (0.27–0.79)	0.43 (0.21–0.65) <sup>b</sup>	0.64 (0.37–0.83)	0.66 (0.42–0.81)		
<b>Disease duration, mo</b>	13.3 (9.2–21.2)	14.4 (10.0–21.7)	11.5 (8.2–15.3)	11.4 (8.0–15.9)		
<b>ALSFRS-R, baseline</b>	41 (36–43)	42 (38–44) <sup>b</sup>	41 (38–44)	41 (38–43)		

Abbreviations: ALS-FTD-Q = Amyotrophic Lateral Sclerosis–Frontotemporal Dementia Questionnaire; ALSFRS-R = ALS Functional Rating Scale–revised; ECAS = Edinburgh Cognitive and Behavioral ALS Screen; FAB = frontal assessment battery; FTD = frontotemporal dementia; VFI = verbal fluency index. Values are median interquartile range (IQR) unless otherwise stated. Values in follow-up columns are calculated based on baseline data. Disease progression rate was calculated as follows: (48-ALSFRS-R)/disease duration (in months). The label of familial ALS was used when there were 2 or more cases of ALS or FTD in the family of the patient.

<sup>a</sup> Missing values n = 3.

<sup>b</sup> Significantly different compared to baseline with *p* < 0.05.

measurements and total ALSFRS-R score are shown (appendix e-1 and figure e-1, doi:10.5061/dryad.8931zcrkv).

### C9orf72-negative ALS

Comparing 268 *C9orf72*-negative patients with ALS with 156 controls at baseline, significantly thinner cortex was found in the primary motor, frontal, and temporal regions (figure 1A). The left accumbens nucleus and right thalamus, bilateral hippocampi, and left amygdala were also significantly smaller in patients (table e-9, doi:10.5061/dryad.8931zcrkv). The inferior lateral and third and fourth ventricles were significantly larger in patients with ALS compared to controls; gray and white matter volumes of the cerebellum were not significantly different (table e-10, doi:10.5061/dryad.8931zcrkv). Baseline connectome analysis revealed the largest connected component of reduced structural connectivity (comprising 140 connections) in the motor network ( $p < 0.001$ ; figure 1C).

At follow-up, additional cortical thinning was found in the bilateral precentral gyri, right paracentral gyrus, and frontal and temporal regions ( $p < 0.05$ , figure 1B). Volumes of the left caudate, right thalamus and accumbens nucleus, right cerebellar cortex, and bilateral hippocampi decreased significantly over time. Volumes of the lateral, third, and fourth ventricles increased significantly over time. Connectome analyses demonstrated a small component (27 connections) with FA reductions over time and none with FA increase ( $p = 0.016$ ; figure 1D).

### C9orf72-positive ALS

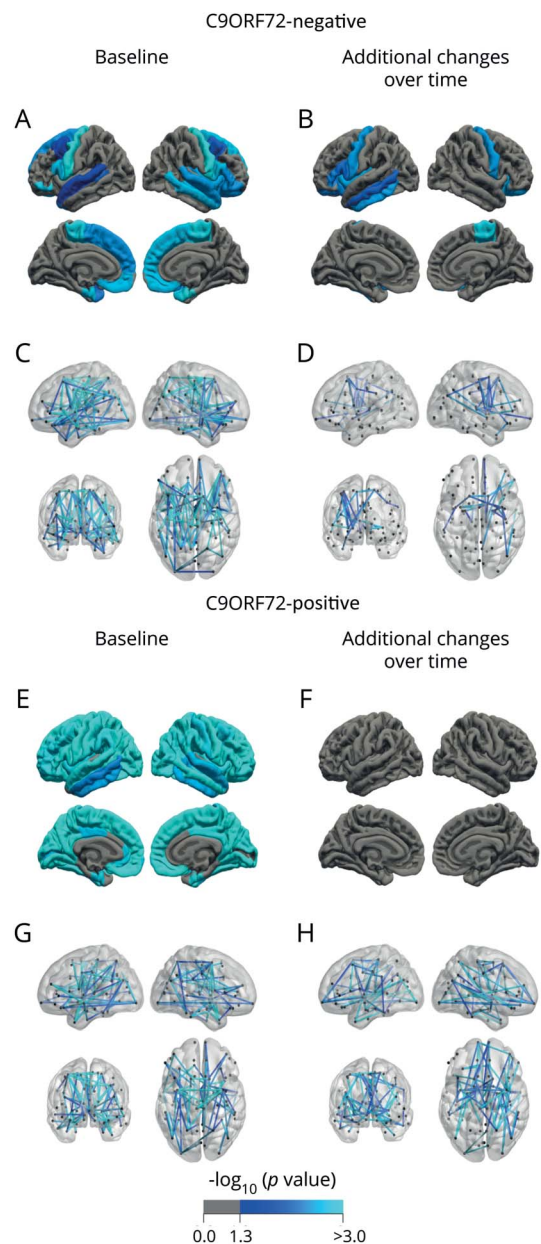
At baseline, more extensive cortical thinning in *C9orf72*-positive patients compared to controls than in the analysis with *C9orf72*-negative ALS was observed (figure 1E). Reduction in volume of the bilateral thalami, caudate, putamen, pallidum, accumbens nuclei, hippocampi, and amygdala and enlargement of the ventricles were found (table e-9, doi:10.5061/dryad.8931zcrkv). Connectome analysis revealed a component of reduced connectivity (86 connections), most notably in connections between the bilateral precentral and paracentral gyri, basal ganglia, and temporal lobe ( $p = 0.005$ , figure 1G).

At follow-up, no additional cortical effects were observed (figure 1F), enlargement of the (inferior) lateral and third ventricles could be detected, and progressive white matter involvement was shown by FA reductions in a large component (71 connections,  $p = 0.048$ , figure 1H).

### Bulbar- vs spinal-onset ALS

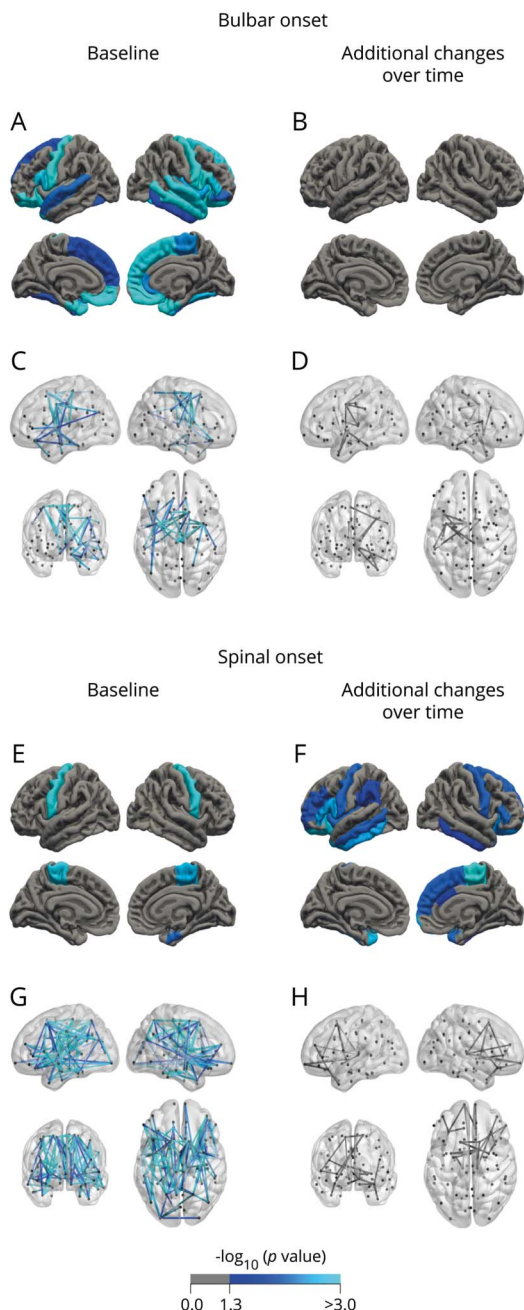
Compared to controls at baseline, *C9orf72*-negative patients with ALS with bulbar onset were found to have more widespread cortical involvement than patients with spinal onset (figure 2, A and E): both onset groups showed thinning of the primary motor regions and the right entorhinal cortex, but bulbar-onset patients also showed thinning of frontotemporal and parietal regions. Regarding subcortical effects, compared

**Figure 1** Brain involvement at baseline and additional changes over time in *C9orf72*-negative and -positive patients with amyotrophic lateral sclerosis (ALS)



(A, B) Comparison of cortical thickness between *C9orf72*-negative patients with ALS ( $n = 268$ ) and controls ( $n = 156$ ). Regions with a significantly lower cortical thickness at baseline (A) and additional cortical changes over time (B) in patients with ALS are marked in blue ( $p < 0.05$ , false discovery rate-corrected). The cortical thickness of each region of interest (ROI) (separately for left and right hemisphere) was taken for the analyses. Each resulting  $p$  value was mapped onto the surface of the entire ROI to indicate significant regional differences between groups. (C, D) Connectome analysis with affected connections in patients with ALS ( $n = 230$ ) compared to controls ( $n = 135$ ) at baseline (140 connections,  $p < 0.001$  [C]), and additional changes over time, centralized in the motor network (D), as expressed by a decrease in fractional anisotropy, marked in blue (27 connections,  $p = 0.016$ ). (E, F) Comparison of cortical thickness between *C9orf72*-positive patients with ALS ( $n = 24$ ) and controls ( $n = 156$ ) at baseline shows widespread cortical involvement (E) and no additional cortical thinning over time (F). (G, H) Connectome analysis reveals widespread connectivity effects in patients with ALS at baseline ( $n = 24$ , 86 connections,  $p = 0.005$  [G]), and extensive additional white matter effects over time (71 connections,  $p = 0.048$  [H]).

**Figure 2** Analysis of cortical thickness and connectivity effects stratified for site of onset



(A–D) Bulbar onset. At baseline, frontotemporal involvement and connectivity effects involving the primary motor regions are shown ( $p < 0.05$  for cortical thickness [A]; 48 connections,  $p = 0.020$  [C]), with no additional cortical changes (B) and only small changes in connectivity over time (11 connections,  $p = 0.063$  [D]). (E–H) Spinal onset. At baseline, cortical thinning is confined to the motor cortex (E), whereas additional cortical changes are found in frontotemporal regions during follow-up (F). Connectome analysis shows a large component of decreased connectivity strength (152 connections,  $p < 0.001$  [G]), again including small changes in the primary motor and frontotemporal regions over time (22 connections,  $p = 0.065$  [H]). Regions unique for bulbar onset were the bilateral fusiform, medial orbitofrontal gyri, pars orbitalis, superior frontal and temporal gyri and temporal lobes, left entorhinal gyrus, right inferior temporal gyrus, insula, middle temporal gyrus, pars opercularis, pars triangularis, rostral anterior cingulate, and the bilateral amygdala. Regions unique for spinal onset were all situated in the left hemisphere: middle and inferior temporal gyri, insula, pars triangularis, and the paracentral gyrus.

to controls, bulbar-onset patients showed larger inferior lateral ventricles and reduced volumes of the bilateral hippocampi, thalami, accumbens nuclei, and right putamen (table e-11, doi:10.5061/dryad.8931zcrkv).

Spinal-onset patients showed larger inferior lateral ventricles and reduced volumes of the bilateral hippocampi compared to controls. Bulbar- and spinal-onset patients showed strongly overlapping effects of the most severely affected connections mainly involving bilateral precentral gyri and paracentral lobules (figure 2, C and G), but the spinal-onset group displayed a more widespread pattern of affected connections (bulbar onset: 48 connections,  $p = 0.020$ ; spinal onset: 152 connections,  $p < 0.001$ ).

No additional changes could be detected in bulbar-onset patients (figure 2, B and D). In contrast, patients with a spinal onset showed progressive cortical thinning in the primary motor and frontotemporal regions, additional decrease in volumes of the bilateral hippocampi, left caudate, right thalamus, and accumbens nucleus, and enlargement of the ventricles, as well as a nonsignificant component of decreased connectivity over time, mainly involving frontotemporal regions (22 connections,  $p = 0.065$ , figure 2, F and H).

### Cognition and behavior

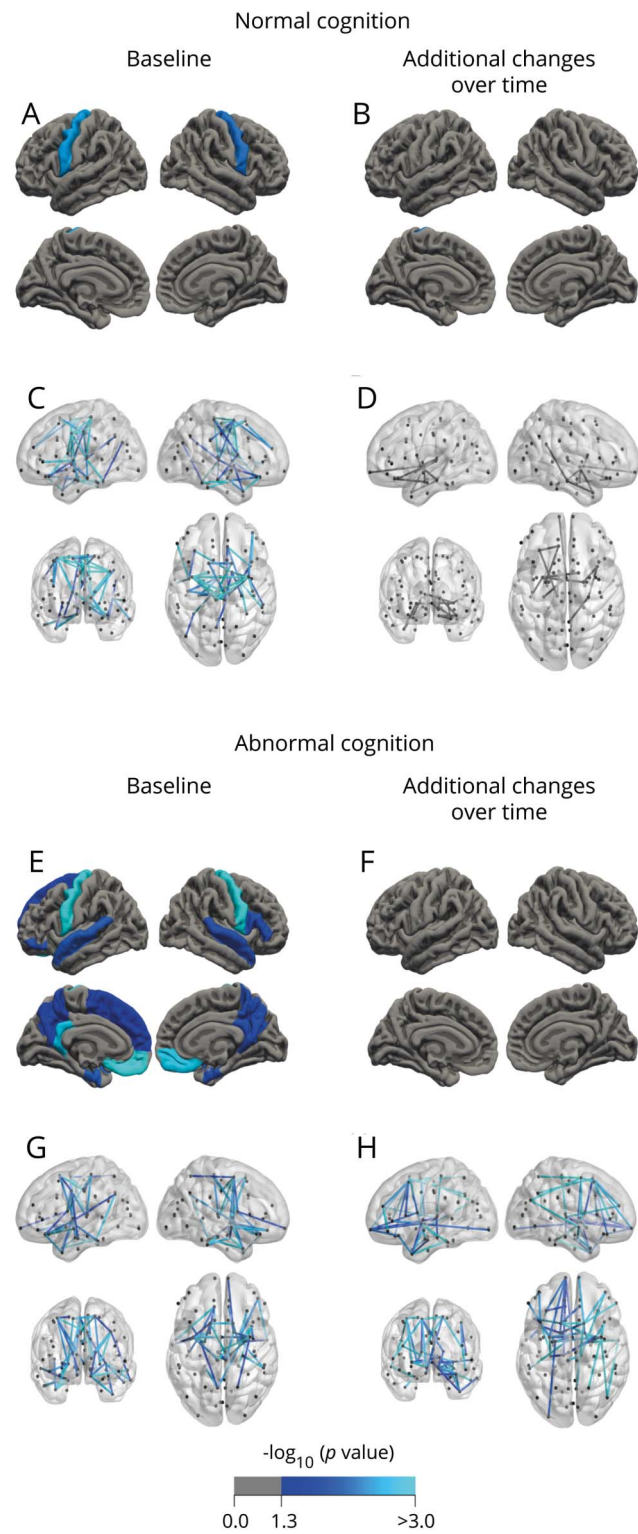
In patients with unaffected cognition or behavior, cerebral changes were mainly characterized by thinner precentral gyri (figures 3A and 4A). The inferior lateral ventricles were enlarged in both groups, and smaller bilateral hippocampi were found for patients with unaffected cognition (tables e-12 and e-13, doi:10.5061/dryad.8931zcrkv). The largest connected component in cognitively or behaviorally unaffected patients was centralized around the motor system (cognition: 44 connections,  $p = 0.026$ , figure 3C; behavior: 51 connections,  $p = 0.021$ , figure 4C).

At follow-up, there was no additional gray or white matter involvement for cognitively or behaviorally unaffected patients; only patients with unaffected behavior showed a smaller right accumbens nucleus and both groups displayed enlarged ventricles.

At baseline, cognitively impaired patients had thinner bilateral precentral gyri and frontotemporal regions (figure 3E) and showed smaller volumes of the bilateral amygdala, hippocampi, caudate, left accumbens nucleus, and right putamen, and enlargement of the inferior lateral and third ventricles (table e-12, doi:10.5061/dryad.8931zcrkv). The largest connected component included frontal and temporal connections (46 connections,  $p = 0.023$ , figure 3G).

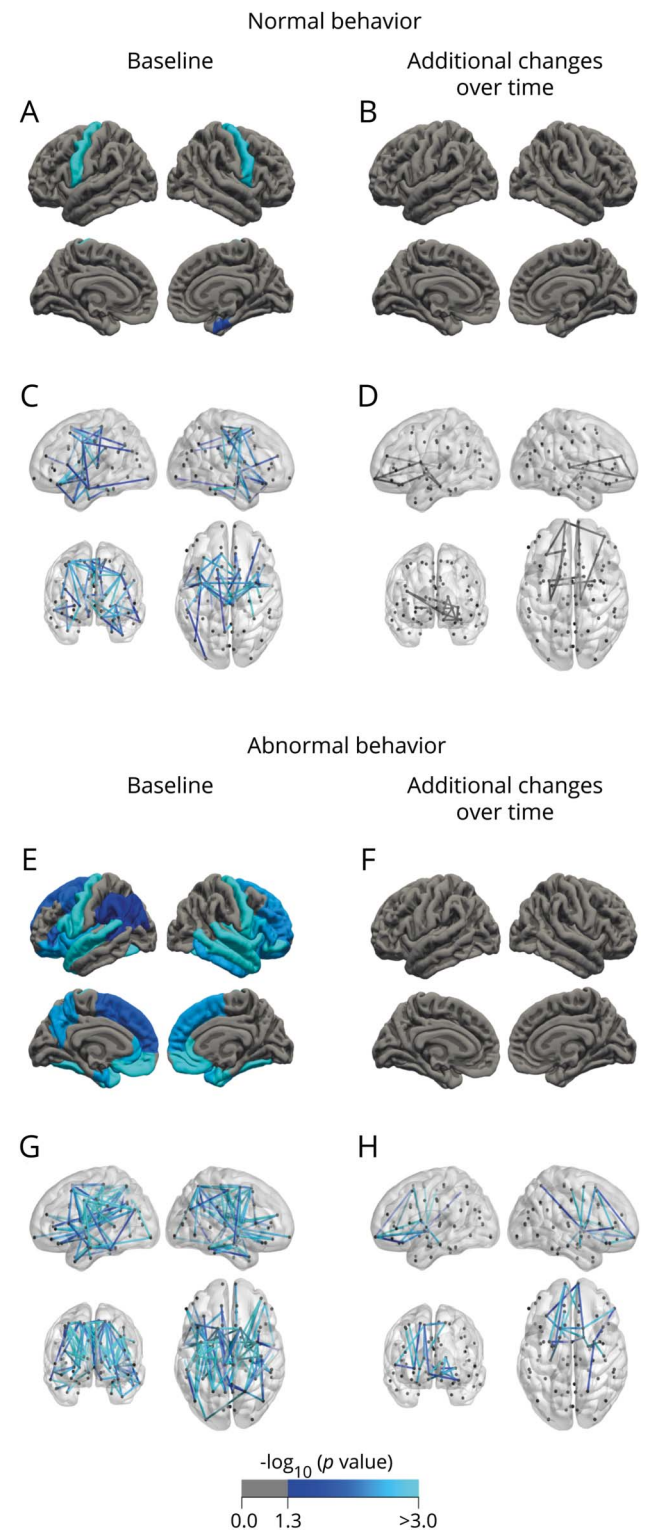
At follow-up, there was no additional gray matter thinning in cognitively impaired patients. They showed decreasing bilateral hippocampi and left caudate volumes and enlarging

**Figure 3** Analysis of cortical thickness and connectivity effects in patients with normal and abnormal cognition



Patients with normal cognition show predominantly involvement of the motor system at baseline (A and C) and minor cerebral change over time (B and D). In contrast, cerebral changes were widespread for gray and white matter and included extramotor regions in patients with abnormal cognition (E and G). Over time, these patients only showed loss of white matter integrity (47 connections,  $p = 0.043$  [F and H]).

**Figure 4** Analysis of cortical thickness and connectivity effects in patients with normal and abnormal behavior



Patients with normal behavior show predominantly involvement of the motor system at baseline (A and C) and minor cerebral change over time (B and D). In contrast, patients with abnormal behavior demonstrated widespread cerebral changes comprising nearly the entire temporal and frontal lobes (E and G). Over time, behaviorally impaired patients showed additional loss of white matter integrity (22 connections,  $p = 0.021$  [F and H]).

ventricles. The significant largest connected component of reduced structural connectivity included frontotemporal regions (47 connections,  $p = 0.043$ , figure 3H).

Patients with impaired behavior showed thinner bilateral precentral gyri, frontal, parietal, and temporal regions, and reduced volumes of the bilateral hippocampi, amygdala, accumbens nuclei, right thalamus, putamen, and caudate, and enlarged inferior lateral and third ventricles (table e-13, doi: 10.5061/dryad.8931zcrkv). The significant largest connected component included motor, temporal, frontal, and parietal connections (110 connections,  $p < 0.001$ , figure 4G).

At follow-up, these patients showed no additional thinning, but ventricular volumes increased. The significant largest connected component included frontal connections (22 connections,  $p = 0.021$ , figure 4H).

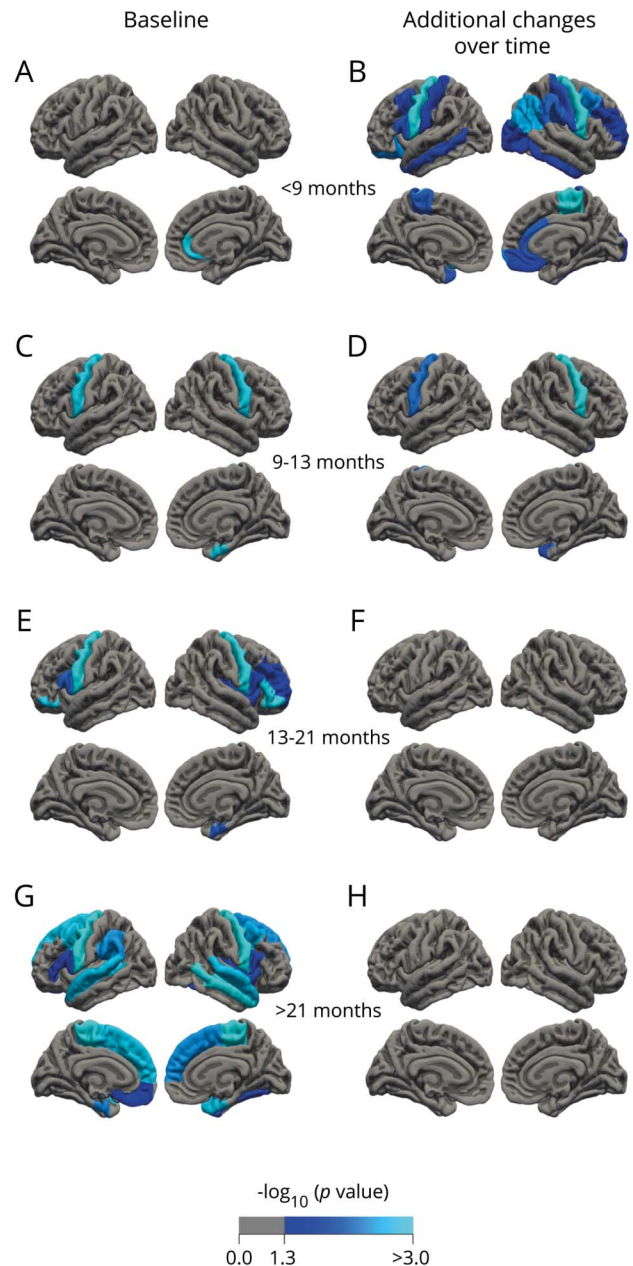
### Effect of disease duration

Patients were compared between quartile groups of disease durations. The first quartile group had a higher progression rate at baseline compared to the others (i.e., 0.78 compared to 0.62, 0.52, and 0.26, respectively).

At baseline, patients with a disease duration of less than 9 months from symptom onset (first quartile group) showed significant thinning of the rostral anterior cingulate cortex (figure 5A); the second quartile group showed cortical thinning in the bilateral precentral gyri and right entorhinal cortex (figure 5C); the third quartile group showed cortical thinning congruent with the second quartile group and thinning of the bilateral pars orbitalis, pars opercularis, right pars triangularis, and rostral middle frontal gyrus (figure 5E); the fourth quartile group showed the most extensive cortical thinning including primary motor regions and frontotemporal regions (figure 5G). Regarding subcortical structures, there was significant enlargement of the inferior lateral ventricles for all quartiles compared to controls (table e-14, doi:10.5061/dryad.8931zcrkv). The first quartile also showed reduced volumes of the left hippocampus and amygdala. Subcortical effects were found in the second quartile (bilateral hippocampi, right pallidum, amygdala, and thalamus and third ventricle) and the fourth quartile (right hippocampus and fourth ventricle). Baseline connectome analyses of the quartile groups showed consistent involvement of the motor network (precentral, paracentral, and brainstem) and limited extramotor involvement (first quartile group: 65 connections,  $p = 0.002$ ; second quartile group: 68 connections,  $p = 0.012$ ; third quartile group: 98 connections,  $p = 0.003$ ; and fourth quartile group: 121 connections,  $p < 0.001$ , figure 6, A, C, E, and G).

Patients with a disease duration of less than 9 months from symptom onset showed significant additional cortical thinning over time, including primary motor and frontotemporal regions (figure 5B). The second quartile group displayed significant additional changes restricted to the bilateral

**Figure 5** Analysis of cortical thickness stratified for disease duration



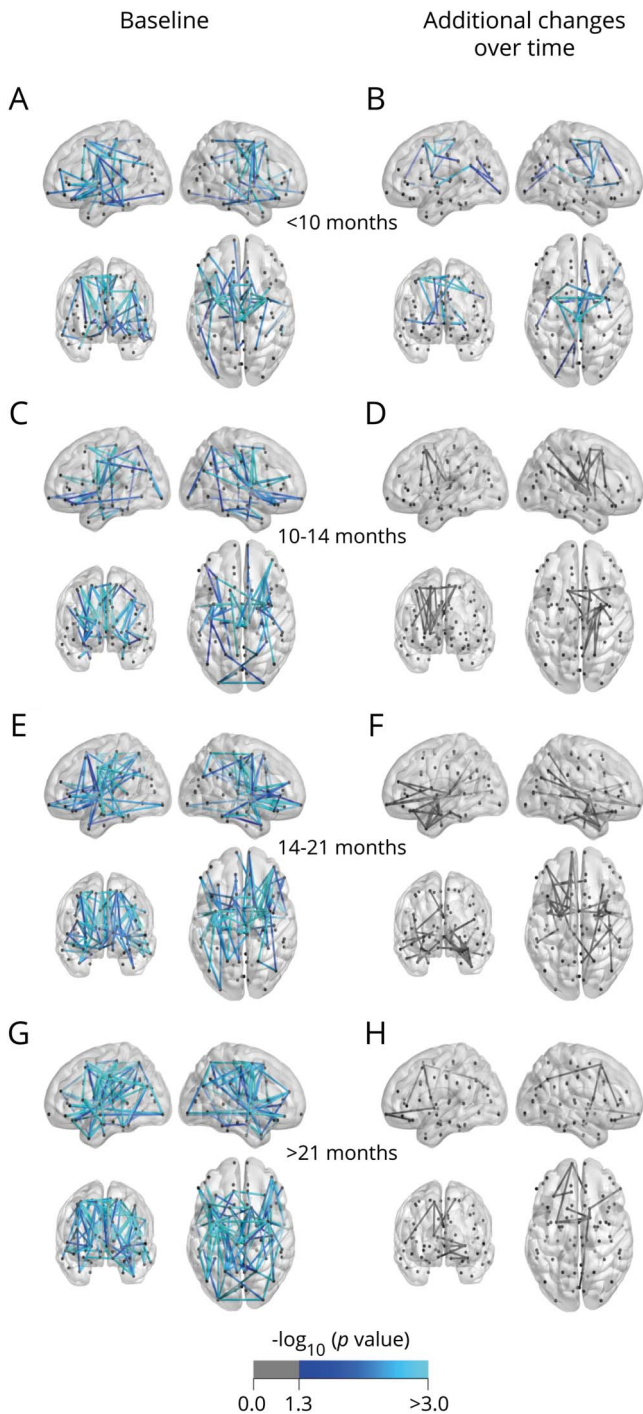
(A, C, E, and G) Baseline. (B, D, F, and H) Additional effects over time. Patients with amyotrophic lateral sclerosis (ALS) with a short disease duration show cortical thinning limited to the rostral anterior cingulate cortex at baseline (A) and additional cortical changes over time in motor and frontotemporal regions (B). Patients with a longer disease duration show cortical involvement of similar frontotemporal regions at baseline and no progressive cortical changes during follow-up.

precentral gyri and right temporal pole (figure 5D). The third and fourth quartile groups showed no significant additional longitudinal cortical changes (figure 5, F and H).

At follow-up, the first quartile group showed an additional decrease in subcortical volumes of the bilateral thalami and hippocampi, left caudate, and right accumbens nucleus, and



**Figure 6** Analysis of connectivity stratified for disease duration



(A, C, E, and G) Baseline. (B, D, F, and H) Additional effects over time. Patients with a long disease duration show extensive connectivity effects at baseline (121 connections,  $p < 0.001$  [G]) and no significant additional effects over time (H). Patients with a short disease duration show effects centered around the motor network at baseline (65 connections,  $p = 0.002$  [A]) and additional connectivity effects over time (23 connections,  $p < 0.001$  [B]).

enlargement of the ventricles. The second and third quartile groups only showed ventricular enlargement over time. No additional effects were detected in the fourth quartile group. Patients with the shortest disease duration were the only

disease duration subgroup that showed significant additional connectivity effects over time (first quartile: 23 connections,  $p < 0.001$ ; second quartile:  $p = 0.070$ ; third quartile:  $p = 0.056$ ; fourth quartile:  $p = 0.436$ , figure 6, B, D, F, and H).

Because patients with an early MRI scan have a faster disease progression, we further explored the effect of disease progression on cerebral changes. The progressive loss of gray and white matter integrity in patients with shorter disease durations (<13 months) is independent of progression rate (appendix e-2, table e-15, and figures e-5 and e-6, doi:10.5061/dryad.8931zcrkv).

### Sensitivity analyses

Sensitivity analyses, excluding participants with an abnormal ECAS score or a pathogenic mutation other than *C9orf72*, showed similar results to those described above. In most subgroups, patients without follow-up data displayed more cerebral involvement than patients with longitudinal data, with considerable overlap of the involved regions (figure e-2, doi:10.5061/dryad.8931zcrkv). Disease effects measured in mean diffusivity are similar to FA: frontal connections are present more frequently in the largest connected components (appendix e-3 and figure e-3, doi:10.5061/dryad.8931zcrkv). Figure e-4 (doi:10.5061/dryad.8931zcrkv) displays direct comparisons between subgroups (appendix e-4, doi:10.5061/dryad.8931zcrkv).

### Discussion

We investigated whether structural MRI could serve as a biomarker of disease progression in clinical trials. We applied multiple modalities of brain imaging, cross-sectionally at baseline and longitudinally, to a large cohort of patients with ALS and controls. Longitudinal effects in the total group of patients were detected in terms of progressive cortical atrophy in primary motor and frontotemporal regions, shrinking subcortical volumes, ventricle enlargement, and loss of white matter integrity in a connected component around the motor cortex. We were also able to identify differential patterns of gray and white matter involvement in subgroups of patients with specific clinical characteristics, disease duration, or genotype. The heterogeneity of our findings among subgroups of patients may have important implications for cross-sectional and follow-up MRI studies in ALS, and the timing of neuroimaging in the disease course for clinical trials and follow-up studies.

We performed a comprehensive study using cross-sectional, longitudinal, and multimodal information. In the LME models, we could use all 292 baseline scans of patients with ALS as well as the available follow-up scans from 150 patients. Thereby, we account for between-subject variation and attrition bias, and thus improve accuracy of the longitudinal analyses, as was reported previously.<sup>28</sup> A limitation of all imaging studies in ALS, including the present study, may be selection bias towards a cohort of patients able to undergo MRI scanning. In our study, this limitation applies in particular to the longitudinal part: these patients less frequently had

a bulbar onset and a slower disease progression than those with only 1 MRI scan. However, by closely monitoring this possible bias during inclusion of patients, we were able, for the first time, to analyze MRI scans of a relatively large number of patients with fast-progressing disease and patients at an early stage of the disease. At baseline, more extensive cerebral involvement was observed in patients with a single scan compared to patients with follow-up scans (figure e-2, doi:10.5061/dryad.8931zcrkv), suggesting that the longitudinal results might be slightly underestimating the extent of brain involvement over time. However, it is also possible that patients with more severe baseline involvement who could not undergo follow-up might have reached a plateau of neurodegeneration. MRI scanners allowing scanning in an upright position may facilitate inclusion of patients with ALS in follow-up studies.

The multiple modalities comprised gray matter data, in terms of cortical thickness and subcortical volumes, and all white matter tracts. Previous multimodal imaging studies included a maximum of 64 patients who were assessed at a single time point. These studies reported both gray and white matter alterations in patients with ALS compared to controls, most prominent in the motor cortex, frontal lobe, and corticospinal tract.<sup>32–35</sup> Our study demonstrates, probably due to the larger sample size, that, besides these regions, temporal regions are exposed to cortical thinning, subcortical structures (i.e., basal ganglia, thalamus, and hippocampi) have reduced volumes, and ventricles are enlarged. By applying connectome-based analyses, we showed widespread loss of white matter integrity, not restricted to the corticospinal tract. Future research could examine cerebellum connectomes to investigate disease effects in this region.

Few previous studies combined a multimodal and longitudinal approach.<sup>32–34</sup> Other longitudinal imaging studies included a single modality (gray or white matter) to examine the pattern of disease progression<sup>36–40</sup> and revealed mainly progressive gray matter atrophy in the primary motor system and frontal areas.<sup>32–34,36</sup> We also found progressive thinning in temporal regions, shrinking of subcortical volumes, and enlarging ventricles at follow-up. Concerning white matter, longitudinal studies focused mainly on the corticospinal tract, showing minor or no changes,<sup>32–34,37,38,40</sup> while assessment of the entire connectome in our study demonstrated significant loss of connectivity in the motor system and its linked connections in the right hemisphere in the absence of increasing FA. Importantly, we showed that progressive loss of gray and white matter integrity typically takes place in patients with shorter disease duration (<13 months after disease onset for gray matter and <10 months for white matter), while previous studies included patients with a mean disease duration at baseline at a later stage of at least 18 months. Loss of white matter integrity in previous studies may thus have already occurred before patients were included, indicating the relevance of timing of neuroimaging. Patients with the shortest disease duration revealed a distinct connectivity profile and

more extensive changes in cortical thickness over time, while patients with longer disease duration showed widespread cortical thinning and loss of connectivity only at baseline, possibly reflecting a kind of brain change end stage. Previous research has shown, by applying simulations, that the healthy connectome provides an anatomical underlying substrate for TDP-43 pathology,<sup>41</sup> following the stages suggested by the Braak model of corticofugal spread.<sup>42</sup> Other studies indicated that interconnected gray matter regions become gradually affected instead of being subject to contiguous spread.<sup>10,43</sup> Future research could, therefore, examine differences between the Braak stages and observed disease spread in patients. As patients with early diagnosis may have a more aggressive disease course,<sup>44</sup> we investigated whether the observed effects reflect a more progressive disease course, but no clear relation between neuroimaging effects and disease progression was found in patients with short or long disease duration. Hence, scanning patients relatively early in the disease course may be more important than stratifying for progression rate. Using mean diffusivity instead of FA might help prolong the opportunity to reveal disease effects in white matter.

Patterns of brain abnormalities may also be determined by site of disease onset. We observed that both bulbar- and spinal-onset subgroups develop atrophy of similar anatomical regions, which is congruent to neuropathologic studies.<sup>45</sup> However, we found a differential pattern of cerebral involvement in patients with bulbar or spinal onset at baseline and at follow-up: bulbar-onset patients showed prominent gray matter atrophy and some white matter changes at baseline, whereas spinal-onset patients mainly displayed widespread loss of connectivity. At follow-up, gray matter became affected in spinal-onset patients, whereas bulbar-onset patients showed no additional brain changes. One could speculate that the prominent white matter changes at baseline, followed by additional gray matter involvement in subsequent scans, might imply a dying-backward process of upper motor neuron degeneration in spinal-onset patients. For bulbar-onset patients, the direction of the disease process is less clear; clinically, upper motor neuron features in the bulbar region are more prominent in the early stage of the disease, implying a dying-forward process in bulbar-onset patients, which is at least not contradicted by our neuroimaging results. Incorporating other phenotypes, such as primary lateral sclerosis or progressive muscular atrophy, might provide more insight in the direction of neurodegeneration.

Previous cross-sectional imaging studies have shown extramotor cerebral involvement of frontal, temporal, and parietal regions in patients with cognitive or behavioral impairment (often considering abnormal cognition and behavior as one impaired group).<sup>35,46–48</sup> In our multimodal study, gray and white matter changes were widespread, mainly in motor and frontotemporal regions, in cognitively and, even more extensively, in behaviorally impaired patients. Subcortical gray matter and white matter changes including frontotemporal regions were found to be progressive over time. In patients

with unaffected cognition and behavior, cerebral changes were restricted to motor regions; thinning of the motor cortex and reduced ventricle volumes were found to be progressive. Thus, these findings largely reflect clinical observations and possibly provide an underlying anatomical substrate for cognitive and behavioral impairment in ALS.

A limitation of our study may be the missing data and heterogeneous methodology for assessing cognitive or behavioral impairment as the ECAS was introduced after the study started. Despite this limitation, we were able to determine, in one of the largest imaging cohorts of cognitively and behaviorally impaired patients with ALS, according to the current Strong criteria,<sup>3</sup> that cognition and behavior are relevant features to consider in follow-up neuroimaging studies in ALS. Future multimodal neuroimaging studies may need to explore more detailed patterns of cognitive and behavioral impairment in a cohort in which cognition and behavior are assessed homogeneously for the entire population.<sup>35</sup> Moreover, it might be worthwhile to incorporate level of education and depression in the analyses, as they have a relevant effect on the brain.<sup>49,50</sup>

A distinctive MRI pattern of cerebral involvement was found in *C9orf72*-positive patients, showing more extensive cortical and subcortical changes, and reduced white matter integrity at baseline, which is in line with previous studies,<sup>11,12</sup> and extensive additional white matter effects over time that were more prominent compared to the effects in *C9orf72*-negative patients. Longitudinal gray matter changes in *C9orf72*-positive patients were limited to enlargement of the ventricles. Our results indicate that connectome analyses, comprising all white matter tracts, may serve as a potential biomarker for upcoming *C9orf72* gene therapy clinical trials. Moreover, the differential patterns of brain involvement emphasize the relevance of screening for the *C9orf72* mutation in neuroimaging studies.

Our study underlines the potential of imaging studies to provide insight into structural brain involvement in relation to the heterogeneity of ALS. By conducting this study in a large cohort of *C9orf72*-positive and -negative patients with ALS, with carefully defined phenotypes and systematic follow-up, including controls, we were able to show differential effects of gray and white matter changes at baseline and during follow-up. The observed results might indicate that cerebral changes in ALS occur at a relatively early stage of the disease, leastwise at the level at which current multimodal techniques are able to detect abnormalities. This may imply that when designing clinical trials, inclusion of patients at an early stage of the disease is necessary to prevent cerebral neurodegeneration. These cerebral changes could be monitored by MRI provided that the appropriate modalities are applied to the right subgroups based on phenotypic characteristics or genotype. Incorporating innovative modalities, such as spinal cord imaging, EEG, or other neurophysiologic techniques, to quantify upper or lower motor neuron involvement in the

analyses of future studies may improve our ability to investigate propagation of neurodegeneration in vivo at different stages of ALS.

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## Disclosure

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<b>Henk-Jan Westeneng, MD</b>	UMC Utrecht, the Netherlands	Design and conception of study, acquisition and analysis of data, drafting the manuscript for intellectual content

## Appendix (continued)

Name	Location	Contribution
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<b>Kevin van Veenhuijzen, MD</b>	UMC Utrecht, the Netherlands	Major role in acquisition of data and preparation of figures
<b>Harold H.G. Tan, MD</b>	UMC Utrecht, the Netherlands	Major role in acquisition of data
<b>Jil M. Meier, PhD</b>	UMC Utrecht, the Netherlands	Major role in acquisition of data, revised the manuscript for intellectual content
<b>Leonhard A. Bakker, MSc</b>	UMC Utrecht, the Netherlands	Major role in acquisition of cognitive data
<b>Jeroen Hendrikse, MD, PhD</b>	UMC Utrecht, the Netherlands	Major role in acquisition of data
<b>Michael A. van Es, MD, PhD</b>	UMC Utrecht, the Netherlands	Design and conception of study
<b>Jan H. Veldink, MD, PhD</b>	UMC Utrecht, the Netherlands	Design and conception of study
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<b>Leonard H. van den Berg, MD, PhD</b>	UMC Utrecht, the Netherlands	Design and conception of study, revised the manuscript for intellectual content

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