Multiparametric MRI study of ALS stratified for the *C9orf72* genotype

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ABSTRACT

Objective: To describe the patterns of cortical and subcortical changes in amyotrophic lateral sclerosis (ALS) stratified for the *C9orf72* genotype.

Methods: A prospective, single-center, single-protocol, gray and white matter magnetic resonance case-control imaging study was undertaken with 30 *C9orf72*-negative patients with ALS, 9 patients with ALS carrying the *C9orf72* hexanucleotide repeat expansion, and 44 healthy controls. Tract-based spatial statistics of multiple white matter diffusion parameters, cortical thickness measurements, and voxel-based morphometry analyses were carried out. All patients underwent comprehensive genetic and neuropsychological profiling.

Results: A congruent pattern of cortical and subcortical involvement was identified in those with the *C9orf72* genotype, affecting fusiform, thalamic, supramarginal, and orbitofrontal regions and the Broca area. White matter abnormalities in the *C9orf72*-negative group were relatively confined to corticospinal and cerebellar pathways with limited extramotor expansion. The body of the corpus callosum and superior motor tracts were affected in both ALS genotypes.

Conclusions: Extensive cortical and subcortical frontotemporal involvement was identified in association with the *C9orf72* genotype, compared to the relatively limited extramotor pathology in patients with *C9orf72*-negative ALS. The distinctive, genotype-specific pathoanatomical patterns are consistent with the neuropsychological profile of the 2 ALS cohorts. Our findings suggest that previously described extramotor changes in ALS could be largely driven by those with the *C9orf72* genotype.

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GLOSSARY

ALS = amyotrophic lateral sclerosis; **C9neg** = patients with amyotrophic lateral sclerosis not carrying the hexanucleotide repeat expansion on *C9orf72*; **C9pos** = patients with amyotrophic lateral sclerosis carrying the hexanucleotide repeat expansion on *C9orf72* (>30 repeats); **DTI** = diffusion tensor imaging; **FA** = fractional anisotropy; **FDR** = false discovery rate; **FOV** = field of view; **FTD** = frontotemporal dementia; **HC** = healthy controls; **MD** = mean diffusivity; **RD** = radial diffusivity; **TBSS** = tract-based spatial statistics; **TE** = echo time; **TFCE** = threshold-free cluster enhancement; **TR** = repetition time; **VBM** = voxel-based morphometry.

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease of heterogeneous clinical phenotypes. An important aspect of disease heterogeneity is the varying degrees of frontotemporal involvement, as evidenced by neuropsychological evaluation,[1] neuroimaging,[2] and postmortem pathologic assessment. The management[3] and survival[4] implications of cognitive and behavioral deficits in ALS are well established, but the etiologic factors leading to frontotemporal dysfunction in ALS are not fully understood. There is increasing evidence[5,6] that the presence of a hexanucleotide expansion in *C9orf72* is a major contributor to frontotemporal pathology in ALS.

Only a small number of imaging studies of patients with expanded *C9orf72* hexanucleotide repeats have been reported to date, many of which have been confined to gray matter[7] analyses or focused primarily on cohorts with frontotemporal dementia (FTD).[8] The most comprehensive neuroanatomical characterization of the *C9orf72* genotype has been provided by detailed postmortem histopathology studies, highlighting genotype-specific changes in the thalamus,[9] hippocampus,[10] and anterior corpus callosum.[9] A distinct neuropathologic signature has been reported in both FTD

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and ALS. In FTD cohorts, *C9Orf72*-positive samples have been successfully distinguished[8] from "tau" and "progranulin." In ALS, samples with *C9orf72* expansion were successfully identified based on p62 immunohistochemistry in the CA4 hippocampal subfield.[11] Motor neuron degeneration[12,9] has been shown in FTD cohorts without antemortem motor symptoms.

As part of a large epidemiology study,[13] our group has previously shown frontotemporal atrophy in *C9orf72* hexanucleotide carriers by performing a preliminary voxel-based morphometry analysis. The objective of this current study is to comprehensively characterize the distinguishing and overlapping neuroimaging signatures of *C9orf72*-negative and C*9orf72*-positive ALS groups.

METHODS

Participants. Thirty-nine patients with ALS and 44 healthy controls were included in this analysis. Patients with ALS were recruited between 2009 and 2011 from the Irish National ALS register, which has been described previously.[14] All patients had probable or definite ALS according to the El Escorial criteria.[15] Clinical and demographic data are summarized in table 1. Patients with ALS and healthy controls were matched for age and education. Patients with ALS positive (C9pos) and negative (C9neg) for the *C9orf72* hexanucleotide repeat expansion were matched for disease duration from onset of symptoms to the date of scan. Healthy controls were unrelated to patients with ALS, had no neurologic or psychiatric disease, and had a negative family history for neurodegenerative conditions. Exclusion criteria for all participants included cerebrovascular disease, traumatic brain injury, or other neurologic or psychiatric illness.

Standard protocol approvals, registrations, and patient consents. All participants provided informed consent in accordance with the medical ethics approval of the research project (Ethics [Medical Research] Committee, Beaumont Hospital, Dublin, Ireland).

Genetics. As described previously,[13] DNA samples were screened by repeat-primed PCR for the presence of a GGGGCC hexanucleotide repeat expansion in *C9orf72* using an Applied Biosystems (Foster City, CA) 3130xl genetic analyzer and visualized using GeneMapper software (version 4.0). Southern blotting samples from 12 individuals carrying the *C9orf72* expansion was used to validate the PCR assay.[13] Patients with more than 30 repeats were considered positive for the hexanucleotide expansion. C9neg and C9pos patients were also screened for mutations in genes previously implicated in ALS, including *FUS, OPTN, SOD1, TARDBP, GRN, ANG,* and *ATXN2*. No previously described mutations were detected in any of these genes in either patient cohort.

Table Demographic profile of participants

Study group	n	Disease duration, mo, mean (SD)	Age, y, mean (SD)	Male/female	Right/left-handed
C9-positive ALS	9	27.5 (10.1)	54.1 (10.4)	7/2	6/3
C9-negative ALS	30	26 (22.1)	59.4 (10.1)	14/16	27/3
Healthy controls	44	Not applicable	60.2 (9.7)	24/20	41/3

Abbreviation: ALS =amyotrophic lateral sclerosis.

MRI. MRI data were acquired on a 3 T Philips Achieva system with gradient strength 80 mT/m and slew rate 200 T/m/s using an 8-channel receive-only head coil. A 3D inversion recovery prepared spoiled gradient recalled echo sequence was used to obtain high-resolution T1-weighted images of the brain, with field of view (FOV) = 256 × 256 × 160 mm, spatial resolution 1 mm^3, repetition time (TR)/echo time (TE) = 8.5/3.9 ms, TI = 1,060 ms, flip angle = 8°, SENSE factor = 1.5, acquisition time = 7 minutes 30 seconds. Diffusion tensor imaging (DTI) was acquired using a spin echoplanar imaging sequence with a 32-direction Stejskal-Tanner diffusion encoding scheme: FOV = 245 × 245 × 150 mm, spatial resolution = 2.5 mm^3, 60 slices with no interslice gap, TR/TE = 7,639/59 ms, SENSE factor = 2.5, b values = 0, 1,100 s/mm^2, with SPIR fat suppression and dynamic stabilization in an acquisition time of 5 minutes 41 seconds.

Imaging analysis. DTI data were preprocessed to provide voxelwise axial diffusivity, fractional anisotropy (FA), mean diffusivity (MD), and radial diffusivity (RD) datasets. Following standard preprocessing steps, statistical analyses of various diffusivity measures were carried out using the tract-based spatial statistics (TBSS) module[16] of the FSL image analysis suite.[17] Study-specific white matter templates and permutation-based nonparametric inference was used for comparison of diffusion parameters between study groups. The threshold-free cluster enhancement (TFCE) method was applied and statistical significance was set at $p < 0.05$ corrected for multiple comparisons across space. t Tests were first carried out between the C9pos and C9neg groups. Subsequently, C9pos vs healthy controls (HC) and C9neg vs HC t tests were performed to identify patterns of genotype-specific white matter vulnerability and white matter regions affected in both ALS cohorts. In order to illustrate the importance of stratifying patients for the presence of the *C9orf72* hexanucleotide repeat expansion, a supplementary DTI analysis was also carried out, where all patients with ALS (n = 39) were placed into a single group irrespective of their *C9orf72* status and comparisons were made to healthy controls (n = 44) for each diffusion parameter. Cortical thickness measurements were carried out with the Freesurfer image analysis suite.[18] Subsequent to standard preprocessing steps,[19] regions of significant cortical thickness loss were explored in the C9pos cohort in comparison to C9neg patients with ALS and healthy controls. False discovery rate (FDR) correction was used to correct for multiple comparisons at $p < 0.05$. We have previously presented a preliminary voxel-based morphometry (VBM) analysis between C9pos and C9neg patients showing cluster-based thresholding results.[13] In a supplementary analysis, we now used the TFCE approach to explore gray matter differences between the study groups. Study-specific gray matter templates and permutation-based nonparametric inference was used as implemented in FSL-VBM.[17]

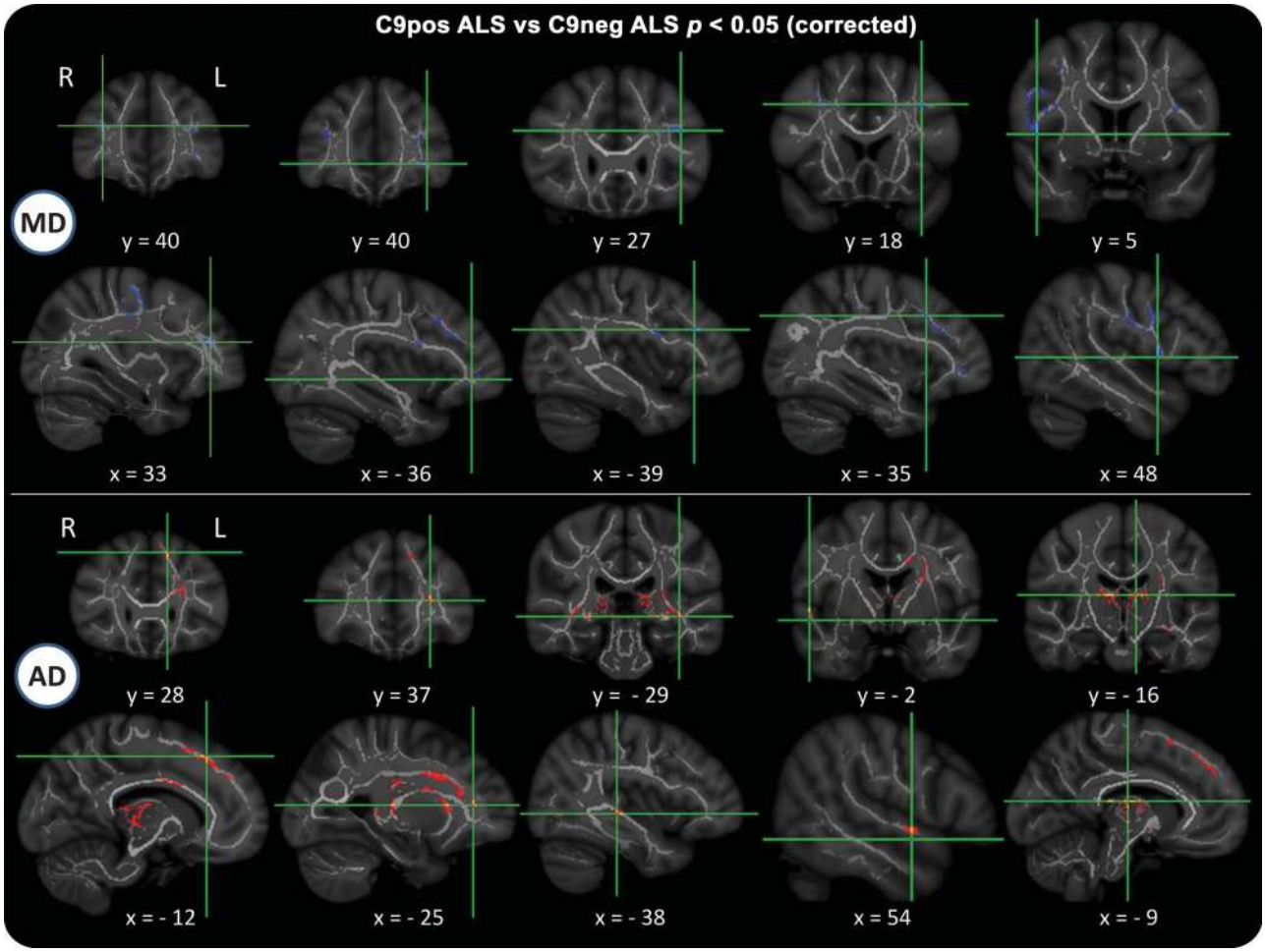
Neuropsychology. Participating patients underwent a comprehensive neuropsychological assessment at the time of the MRI scan. The neuropsychological battery has been described in detail previously.[20,21] Briefly, it included tests for executive function, letter fluency, category fluency, attention, memory, language, visuospatial skills, and behavioral domains. Where possible, tests were corrected for physical disability.[22] Reference psychometric values were provided by a large population-based, age- and education-matched cohort (n = 110) of healthy controls. Based on the corrected scores, all patients were categorized as no impairment (cognitive or behavioral), ALS-FTD (fulfilling the Neary criteria for FTD), executive dysfunction (patients scoring 2 SDs below the mean of healthy controls on at least 2 executive tasks), and nonexecutive impairment (patients with visuospatial, language, or memory impairments).

RESULTS **DTI.** The main objective of the project was to describe genotype-specific patterns of preferential white matter involvement. Differences in various white matter measures between C9pos and C9neg patients with ALS revealed statistically significant clusters for axial and mean diffusivity (figure 1). Discriminating and overlapping pathoanatomical traits of the C9pos and C9neg groups were observed with reference to HC in figure 2. FA, RD, and MD analyses indicate that C9pos patients display mostly frontotemporal abnormalities as opposed the C9neg patients, who show principally motor function–related white matter abnormalities. The overlap between the white matter pathology of the 2 ALS groups is confined to the body of the corpus callosum and the superior corticospinal pathways. Genotype-specific changes were also identified in the genu of the corpus callosum, anterior commissure, and in the bilateral thalami (figure e-1 on the *Neurology*® Web site at www.neurology.org) in association with the *C9orf72* mutation.

Similarly to previous DTI studies, the entire group of patients with ALS (n = 39)—unsegregated based on *C9orf72* status—demonstrated a mixture of motor and frontotemporal tract degeneration (figure e-2). When this is contrasted by genotype-based analyses, it appears that frontotemporal changes are likely to be driven by the C9pos cohort.

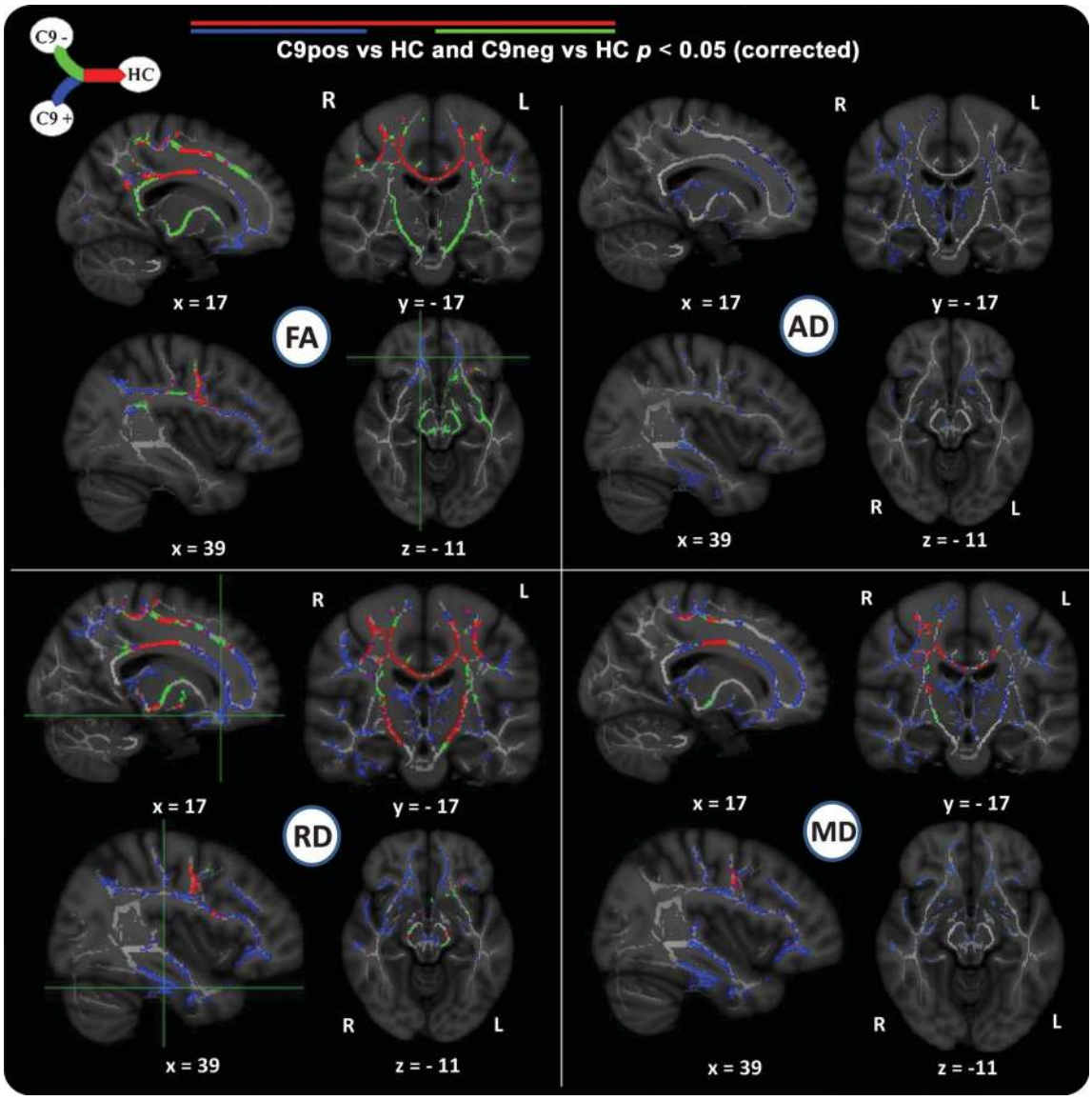
Cortical thickness analyses. Significant focal cortical thickness differences were identified between the C9pos and C9neg cohorts in the left fusiform, left supramarginal, left superior temporal gyrus, left orbitofrontal cortex, left lateral occipital cortex, and left posterior cingulate (figure 3). Cortical thickness differences between the C9neg group and HC did not reach statistical significance following FDR corrections. However, focal cortical thickness differences between the C9pos cohort and healthy controls revealed a strikingly symmetrical pattern of cortical atrophy in the bilateral dorsolateral prefrontal cortices, insular cortices, fusiform gyri, supramarginal cortices, lateral

Figure 1 **Patterns of significant white matter diffusivity differences between patients with ALS carrying the *C9orf72* hexanucleotide repeat expansion and patients with ALS without it**



Highlighted clusters are shown in coronal and sagittal representations and indicated by green crosshairs. Regions of increased mean diffusivity (MD) in the C9pos cohort are highlighted in blue, regions of increased axial diffusivity (AD) in red. The coordinates are with reference to the Montreal Neurological Institute stereotactic template. ALS = amyotrophic lateral sclerosis.

Figure 2 Patterns of white matter pathology in ALS genotypes compared to healthy controls



Blue indicates white matter regions uniquely affected in patients with amyotrophic lateral sclerosis (ALS) carrying the *C9orf72* hexanucleotide repeat expansion. Green shows white matter regions exclusively affected in patients with ALS without the *C9orf72* hexanucleotide repeat. Red indicates the overlap of white matter involvement in both *C9orf72*-negative and *C9orf72*-positive ALS compared to healthy controls (HC). AD = axial diffusivity; FA = fractional anisotropy; MD = mean diffusivity; RD = radial diffusivity.

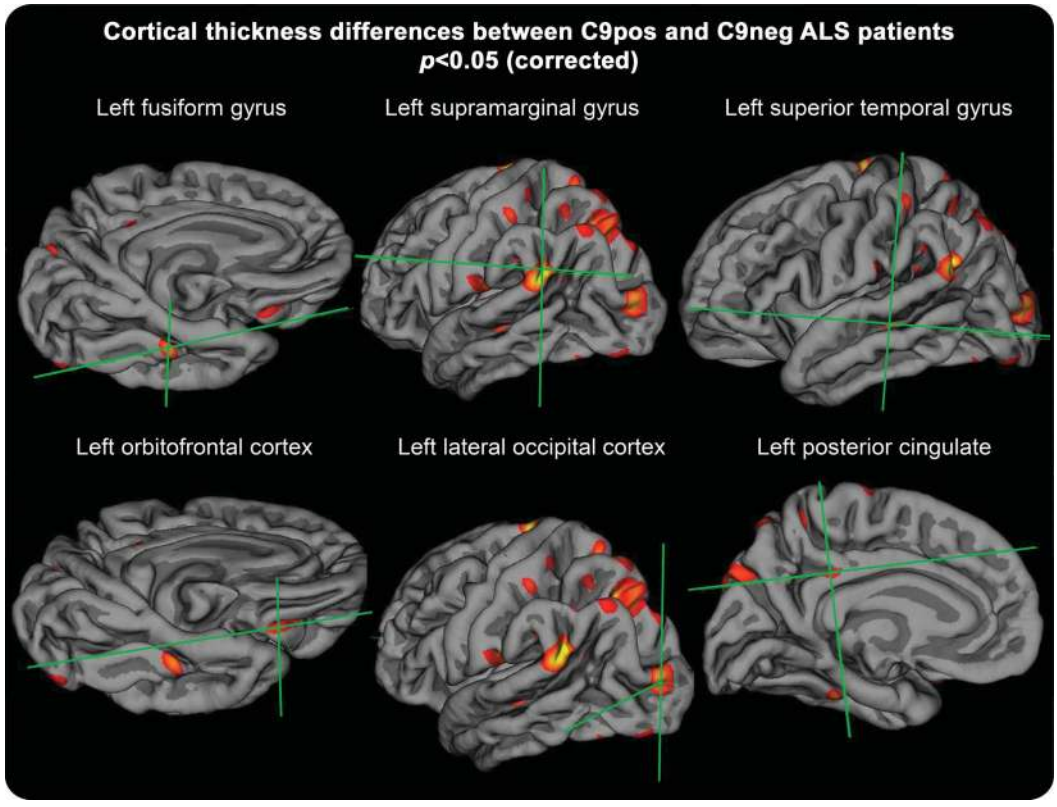
occipital cortices, precuneus, temporal poles, pars triangularis, and pars opercularis of the inferior frontal gyri.

VBM. VBM highlighted extensive orbitofrontal, opercular, and temporal changes in the *C9orf72*-positive cohort compared to HC and C9neg ALS (figure e-3).

Consistency between the various imaging techniques. The various imaging methods reveal a congruent pattern of cortical and subcortical abnormalities in association with the *C9orf72* ALS genotype. This concordance is best demonstrated by cortical and subjacent subcortical changes (figure 4). Additionally, *C9orf72*-specific degeneration in the genu of the corpus callosum or the anterior commissure is consistent with symmetrical gray matter loss in the inferior frontal lobes and temporal poles, respectively.

Neuropsychology. All patients with ALS had undergone detailed neuropsychological assessment. Six (66.6%) of those carrying the *C9orf72* hexanucleotide repeat had evidence of FTD and 2 (22%) of them had executive dysfunction. Conversely, in the *C9orf72*-negative group, only 3 (10%) patients had FTD and 2 (6%) had executive deficits. The remainder were cognitively intact. The differences in the cognitive profile of *C9orf72*-positive and -negative patients were compared using χ^2 test (Fisher exact with Monte Carlo correction) and were statistically significant ($p < 0.0001$). This pattern was broadly reflective of the dichotomous

Figure 3 Key brain regions with significant cortical thickness differences between patients with ALS carrying the *C9orf72* hexanucleotide repeat expansion and patients with ALS without it ($p < 0.05$ corrected for multiple comparisons)



Pial surfaces are shown. ALS = amyotrophic lateral sclerosis.

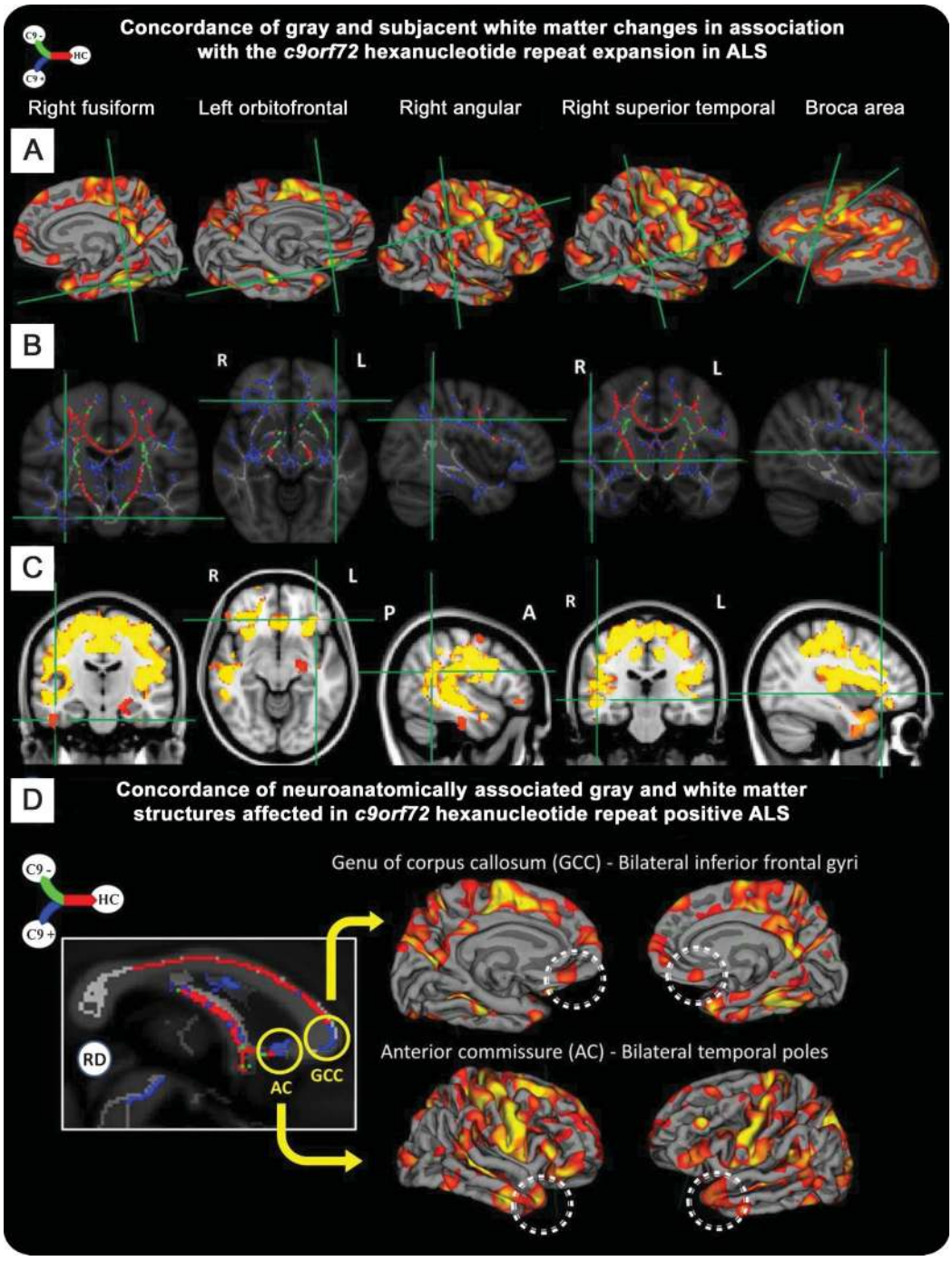
neuropsychological profile of the Irish ALS population.[13]

DISCUSSION The purpose of this study was to examine the imaging characteristics of ALS stratified by *C9orf72* genotype. Our findings suggest a characteristic *C9orf72* repeat expansion–specific neuroimaging signature in ALS, reflecting the higher frequency of cognitive and behavioral impairment within this subgroup.[13] The observed cortical, subcortical, and bithalamic changes identified by this study are entirely consistent with recent postmortem histopathology studies of the *C9orf72* repeat expansion.[9,10] A key finding of our work, however, is that the white matter abnormality seen in patients with ALS without the hexanucleotide repeat expansion is relatively confined to the corticospinal and corticobulbar pathways, representing a "classical," "Charcot type" motor system degeneration with limited extramotor involvement. Similar observations have been made by neuropathology studies[11] describing a relative paucity of extramotor pathology in patients without the hexanucleotide repeat expansion. This reflects previous clinical observations that patients with ALS without the *C9orf72* hexanucleotide repeat expansion are less likely to exhibit evidence of cognitive and behavioral impairment.[13] Taken together, these findings support the contention that the *C9orf72* genotype is an important contributor to extramotor involvement in ALS.

Few imaging studies to date have segregated patients with ALS based on cognitive categories[2] or *C9orf72* status. MRI studies of unsegregated patients with ALS have been inconsistent with respect to extramotor involvement. It is possible that the conflicting reports of extramotor pathology in previous neuroimaging studies in ALS have been partially driven by the inclusion of varying numbers of *C9orf72* hexanucleotide repeat carriers. This is further demonstrated by supplementary figure e-2, where unsegregated and genotype-based TBSS analyses are juxtaposed. The observed distinctive genotype-specific changes make a strong argument for stratification by *C9orf72* status in future imaging, epidemiology, and pharmaceutical studies in ALS.

Although previous reports have described thalamic changes in ALS,[23] their significance has not been widely recognized, possibly due to the absence of convincing clinical and genetic correlations. Our study suggests that bilateral thalamus involvement is an important feature of *C9orf72*-positive ALS (figure e-1). Further classifier studies are warranted, as the thalamus could represent an

Figure 4 Concordance of key *C9orf72*-specific gray and white matter clusters with reference of healthy controls as identified by various imaging techniques



(A) Cortical thickness measurements (C9pos vs healthy controls [HC] $p < 0.05$, false discovery rate corrected). (B) Tract-based spatial statistics (for radial diffusivity: C9pos vs HC $p < 0.05$, threshold-free cluster enhancement [TFCE] corrected). (C) Voxel-based morphometry (C9pos vs HC $p < 0.005$, TFCE corrected). (D) Neuroanatomically associated gray and white matter structures affected in *C9orf72*-positive amyotrophic lateral sclerosis (ALS). A = anterior; P = posterior; RD = radial diffusivity.

important discriminating structure between C9pos and C9neg ALS.

The functional neuropsychological profiles of both patient cohorts strongly support our structural imaging findings. The high proportion of patients with behavioral and executive dysfunction in the C9pos group is not surprising in the context of the extensive orbitofrontal and dorsolateral prefrontal pathology identified by the analyses. Likewise, the low rates of cognitive or behavioral deficits in the C9neg ALS group are consistent with the limited degree of extramotor abnormalities seen on imaging. While traditionally executive dysfunction is regarded as the most common type of cognitive impairment in ALS, high prevalence of language impairment has been

recently established.[24] Our study highlights extensive focal white and gray matter changes in the pars triangularis and pars opercularis regions of the left inferior frontal lobe in association with the *C9orf72* ALS genotype (figure e-1). These regions represent the Broca area for most right-handed and the majority of left-handed people.[25] We suggest that the specific brain regions identified by this study in association with the *C9orf72* genotype should affect the design of future neuropsychological batteries by providing the anatomic bases for targeted assessments, such as in-depth assessments of language domains. Similarly, based on the consistent bilateral fusiform gyrus involvement, tests of face and word recognition should be considered in addition to the widely used executive tests.

Our results demonstrate that in addition to the strong frontotemporal signature of the *C9orf72* genotype, the mutation also contributes to motor neuron degeneration, in particular inferior to the bilateral motor cortices. As illustrated in red in figure 2, the superior motor pathway pathology of the C9pos cohort overlaps with the more extensive corticospinal tract degeneration typical of the C9neg cohorts. This is consistent with histologic evidence of motor neuron degeneration in *C9orf72* repeat expansion carriers in FTD without antemortem motor symptoms[9,12] and raises the question of whether *C9orf72*-positive FTD populations should be regarded as presymptomatic for ALS. Detailed neuroimaging and electrophysiologic studies of *C9orf72*-positive patients with FTD might reveal evidence of early, presymptomatic motor neuron degeneration.

The body of the corpus callosum has long been regarded as an important, consistent site of ALS pathology[26] and this was also observed in our study in patients with ALS irrespective of their *C9orf72* status. As illustrated in yellow in figure e-1, if patients with ALS are not segregated according to *C9orf72* status, corpus callosum involvement can be identified, but the genotype-specific segmental vulnerability is not revealed. As shown in red, the body of the corpus callosum is affected in both genotypes; however, changes in the genu of the corpus callosum and in the anterior commissure are specifically associated with the *C9orf72* hexanucleotide expansion.

The different diffusion parameters used in the study reflect on various aspects of the white matter microstructure. Axial diffusivity is generally regarded as an axonal marker and[27,28] RD as a myelin-related measure.[29,30] FA and MD are histologically less specific, yet sensitive composite markers of white matter integrity. While axial diffusivity (λ_1) and RD ($[\lambda_2 + \lambda_3]/2$) are independent variables, MD is the mean of the 3 diffusion tensor eigenvalues λ_1, λ_2, λ_3 and FA is a measure of anisotropy. Our data suggest that the various diffusivity measures display different sensitivities in highlighting genotype-specific changes. As shown in figure 2, contrasting planes of identical coordinates for the 4 diffusivity measures, it appears that RD captures both the motor tract degeneration associated with the C9neg group as well as frontotemporal changes in the C9pos group. This patchy, mosaic-like, genotype-specific white matter involvement is also demonstrated by the comprehensive FA analysis. The limitation of FA compared to RD is that it fails to highlight *C9orf72*-specific thalamic changes. On the other hand, axial diffusivity seems to preferentially highlight the thalamic and frontotemporal changes in *C9orf72* disease, and does not capture the archetypal corticospinal tract changes of C9neg ALS in the mesencephalon or internal capsules. We therefore suggest that axial diffusivity and RD analyses should be routinely included in future ALS imaging studies in addition to the widely used assessment of FA.

The most important limitation of the presented study is the relatively small number of mutation carriers included. However, the observed changes are fully consistent with the emerging pathology and neuropsychology data on the *C9orf72* hexanucleotide repeat expansion in ALS. Another potential limitation of our study is that PCR technique rather than Southern blotting was used to identify patients with repeat expansions. While Southern blotting provides valuable information on the precise size of the repeat expansion, the sensitivity and specificity of repeat primed PCR to detect pathologic expansions has also been validated.[31] We also acknowledge the high proportion of left-handed patients in our *C9orf72*-positive cohort, which is a function of the relatively small sample size and not reflective of the genotype on a population level. Finally, it is conceivable that our observations may reflect a signature of ALS dementia rather than a signature of the *C9orf72* hexanucleotide repeat. We consider this unlikely as the findings reflect closely the previously described neuropathologic signature of *C9orf72* repeat expansion. Moreover, we and others have shown previously that the majority of patients with the *C9orf72* repeat have evidence of cognitive or behavioral impairment, and 66% of those in this study had evidence of frontotemporal dementia.

We have shown that the *C9orf72* hexanucleotide repeat expansion genotype in ALS has a characteristic imaging profile with a distinctive pattern of cortical, subcortical, and thalamic involvement. The key brain regions include orbitofrontal, fusiform, thalamic, superior temporal regions, and Broca area. The anatomical distribution differs from non-*C9orf72*-associated ALS, in which white matter abnormalities occur primarily within the motor pathways. The findings provide important structural insights into the functional deficits underpinning the clinical phenotype. Given the very distinct anatomical signature of the *C9orf72* ALS genotype, we suggest that future imaging, neuropsychology, and pharmaceutical studies in ALS be stratified for the presence of this mutation. Ultimately, it is likely that multimodal imaging of this genotype will be a sensitive predictor of clinical

phenotype and has the potential to presage cognitive decline in patients with ALS and motor decline in FTD.

AUTHOR CONTRIBUTIONS

Peter Bede: conceptualization and design of the study, imaging data acquisition, neuropsychological testing, analysis and interpretation of MRI data, drafting the manuscript. Arun L.W. Bokde: contribution to the analysis of the MRI data, revision of the manuscript for intellectual content. Susan Byrne: genetic analysis for *C9orf72* hexanucleotide repeat, interpretation of genetic data, revision of the manuscript for intellectual content. Marwa Elamin: analysis and interpretation of neuropsychological data, revision of the manuscript for intellectual content. Russell McLaughlin: contribution to the study design, optimization of MRI protocols, genetic analysis for known ALS mutations such as *FUS, OPTN, SOD1, TARDBP, GRN, ANG,* and *ATXN2*. Kevin Kenna: contribution to the study design, genetic analysis for known ALS mutations such as *FUS, OPTN, SOD1, TARDBP, GRN, ANG,* and *ATXN2*. Andrew J. Fagan: contribution to the biophysical aspects of the study, development and optimization of MRI protocols and sequences. Niall Pender: supervision of the neuropsychological aspects of the study. Daniel G. Bradley: supervision of all genetic aspects of the study. Orla Hardiman: principal investigator, Director of the Irish ALS Register and the Irish National ALS Clinic, supervision of all aspects of the study, drafting and revising the manuscript.

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DISCLOSURE

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