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Neuroimaging correlates of disability in Motor Neuron Disease

A Master thesis submitted to Trinity College Dublin for the degree
of M.Sc. in Clinical Medicine

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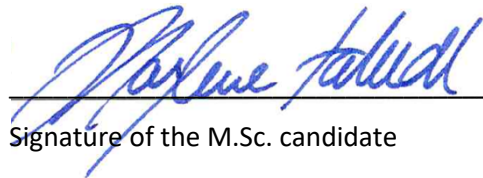
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Publications arising from this course

1. **Tahedi, M.**, Tan, E. L., Shing, S. L. H., Chipika, R. H., Hengeveld, J. C., Vajda, A., Doherty, M. A., McLaughlin, R. L., Siah, W. F., Hardiman, O., & Bede, P. (2023). Brainstem-cortex disconnection in amyotrophic lateral sclerosis: bulbar impairment, genotype associations, asymptomatic changes and biomarker opportunities. *Journal of Neurology*.

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2. **Tahedi, M.**, Tan, E. L., Chipika, R. H., Lope, J., Hengeveld, J. C., Doherty, M. A., McLaughlin, R. L., Hardiman, O., Hutchinson, S., McKenna, M. C., & Bede, P. The involvement of language-associated networks, tracts and cortical regions along the ALS-FTD spectrum: a structural-functional computational neuroimaging study. *Brain and Behavior*, under peer-review.

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3. **Tahedi, M.**, Tan, E. L., Shing, S. L. H., Chipika, R. H., Siah, W. F., Hengeveld, J. C., McLaughlin, R. L., Hardiman, O., & Bede, P. (2023). Not a benign motor neuron disease: longitudinal imaging captures relentless motor connectome disintegration in Primary Lateral Sclerosis. *European Journal of Neurology*.

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4. **Tahedi M.**, Tan E. L., Siah, W. F. , Hengeveld J. C., Doherty, M. A., McLaughlin, R. L., Hardiman, O., Finegan, E., & Bede, P. Radiological correlates of pseudobulbar affect: Corticobulbar and cerebellar components in primary lateral sclerosis. *Journal of the Neurological Sciences*, 451, 120726.

→ Running title *“Pseudobulbar affect in PLS”*

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Abbreviations

AD: axial diffusivity, **AF:** arcuate fascicle, **AFNI:** Analysis of Functional NeuroImages, **ALS:** amyotrophic lateral sclerosis, **ALSFRS-r:** revised amyotrophic lateral sclerosis functional rating scale, **ANOVA:** analysis of variance, **AUC:** area under the curve, **BA:** “bulbar asymptomatic”- patients with spinal onset disease without bulbar symptoms who tested negative for *C9orf72* repeat expansions, **BrS:** Brainstem shape, **BOLD:** blood-oxygen-level-dependent (BOLD) signal, **BS:** “bulbar symptomatic”- patients with bulbar symptoms, **Bul:** bulbar segment of the primary motor cortex, **bvPPA:** behavioral variant primary progressive aphasia, **C9+:** ALS patients with GGGGCC hexanucleotide repeat expansion in *C9orf72*, **C9-:** ALS patients without GGGGCC hexanucleotide repeat expansion in *C9orf72*, **C9orf72:** chromosome 9 open reading frame 72, **CBT:** corticobulbar tract, **CC:** corpus callosum, **CERES:** CEREBellum Segmentation, **CoSMoMVPA:** Multi-Modal Multivariate Pattern Analysis, **CSD:** constrained spherical deconvolution, **CST:** corticospinal tract, **CT:** cortical thickness, **DC:** disease control, **DTI:** diffusion tensor imaging, **DWI:** diffusion-weighted imaging, **ECAS:** Edinburgh Cognitive and Behavioural ALS Screen, **EL:** emotional lability, **ELQ:** emotional lability questionnaire, **EMG:** electromyography, **EMM:** estimated marginal mean, **EPI:** echo-planar imaging, **FA:** fractional anisotropy, **FC:** functional connectivity, **FLAIR:** fluid-attenuated inversion recovery, **fMRI:** functional MRI, **fODF:** fibre orientation distribution function, **FOV:** field of view, **FrSBe:** Frontal Systems Behavior Scale, **FSL:** FMRIB’s Software Library, **FTD:** frontotemporal dementia, **FWE:** familywise error, **GM:** grey matter, **HADS:** Hospital Anxiety and Depression Scale, **HARDI:** High Angular Resolution Diffusion Imaging, **HC:** healthy control, **HCP:** Human Connectome Project, **HRE:** hexanucleotide repeat expansion, **HSP:** hereditary spastic paraplegia, **ICBM:** International Consortium for Brain Mapping, **ICP:** inferior cerebellar peduncle, **IEED:** involuntary emotional expression disorder, **IFO:** inferior occipito-frontal fascicle, **ILF:** inferior longitudinal fascicle, **IR-SPGR:** inversion recovery prepared spoiled gradient recalled echo, **IQR:** interquartile range, **IR-TSE:** inversion recovery turbo spin echo sequence, **JHU:** Johns Hopkins University, **LH:** left hemisphere, **LL:** lower limb, **LMN:** lower motor neuron, **Lt:** Left, **M1:** primary motor cortex, **MCP:** middle cerebellar peduncle, **ML:** machine-learning, **MND:** Motor neuron disease, **MNI152:** Montreal Neurological Institute 152 standard space, **MRS:** MR spectroscopy, **MS:** multiple sclerosis, **MSA-C:** multiple system-atrophy-cerebellar type, **MUNE:** Motor unit number estimation, **MUNIX:** motor unit number index, **MV:** medullary volume, **NCI:** non-cognitively impaired, **nfvPPA:** non-fluent variant primary progressive aphasia, **NISALS:** Neuroimaging Society in ALS, **NODDI:** neurite orientation dispersion and density imaging, **PBA:**

pseudobulbar affect, **PCL**: pathological crying and laughing, **PCR**: polymerase chain reaction, **PLS**: Primary lateral sclerosis, **PM**: premotor area, **PMC**: primary motor cortex, **PUMS**: Penn Upper Motor Neuron Score, **QC**: quality control, **QSM**: quantitative susceptibility mapping, **RH**: right hemisphere, **RD**: radial diffusivity, **ReHo**: regional homogeneity, **ROC**: receiver operator characteristic curve, **ROI**: region of interest, **rs-fMRI**: resting-state functional MRI, **Rt**: right, **SBMA**: spinal-bulbar muscular atrophy, **SC**: structural connectivity, **SCP**: superior cerebellar peduncle, **SD**: standard deviation, **SE-EPI**: spin-echo echo planar imaging, **SENSE**: sensitivity encoding, **SLF**: superior longitudinal fascicle, **SMA**: spinal muscular atrophy, **SPIR**: spectral presaturation with inversion recovery, **SSRIs**: selective serotonin reuptake inhibitors, **T**: Tesla, **T1w**: T1-weighted imaging, **TAP20**: number of taps in 20sec, **TBI**: traumatic brain injury, **TBSS**: tract-based spatial statistics, **TCA**: tricyclic antidepressants, **TE**: echo time, **TFCE**: threshold-free cluster enhancement, **TI**: inversion time, **TIV**: total intracranial volume, **TMS**: transcranial magnetic stimulation, **TR**: repetition time, **Tukey HSD tests**: Tukey's Honest Significant Difference test, **UF**: uncinata fascicle, **UL**: upper limb, **UMN**: upper motor neuron, **svPPA**: semantic variant primary progressive aphasia, **WES**: whole exome sequencing, **WM**: white matter, **XTRACT**: cross-species tractography

1 Summary

The current thesis is comprised of four peer-reviewed articles. In this section, a summary of these articles is provided. This summary focuses on explaining the (1) cohesive theme, (2) rationale, (3) novelty, (4) main objectives, and (5) clinical relevance of the synopsis of the four peer-reviewed papers. At the end of this summary, a reference list with the details of the published outputs is provided.

1.1 Cohesive theme

Motor neuron diseases (MND) clinically present with a wide variety of motor and extra-motor symptoms. However, the pathological correlates of such symptom manifestation and their progression is poorly understood. In the series of studies comprising this thesis, we used novel multimodal neuroimaging metrics to characterise both the cross-sectional and longitudinal manifestation of both types of neurological deficits. We investigated multimodal MRI data from different types of MND disorders, including ALS (and ALS variants, such as ALS-FTD), and PLS, and investigated derived MRI metrics both at baseline and their progression across multiple follow-up assessments. To understand the potential clinical impact of the observed changes, we used rich neuropsychological and clinical assessments to analyze associations. In all studies, multimodal neuroimaging metrics were investigated in MND, whereas the first two studies focus on ALS (and variants) as the most common MND disorder, and the remaining two studies focus on PLS as a relatively rare MND. Whereas the motor system was targeted in the first two studies, extra-motor systems were targeted in the remaining two studies.

1.2 Rationale

The pathological substrates of the manifestation and progression of motor and extra-motor symptoms is poorly understood in MND. Effective therapy is hardly available for symptom alleviation, and extra-motor involvement in particular is hardly appraised in clinical trials. However, their presence severely impacts patients' quality of life and patient management for caregivers and -providers. With the introduction of advanced multimodal neuroimaging metrics into MND studies, novel candidate biomarkers can be identified, with which such deficits can be monitored in clinical trials and in turn motivate the development of therapeutics specifically targeted at such quality-of-life-limiting disability.

1.3 Novelty

This series of studies is distinguished from existing literature by three main novelties, comprising the (1) applied methods, (2) the investigated study populations and (3) the available rich clinical, genetic and neuropsychological characterisation of the study populations: Concerning (1), the applied method: Novel multimodal neuroimaging metrics were calculated from the MND study populations, including (i) metrics estimating atrophy, via cortical thickness and volumetry estimations of cerebral and cerebellar regions, calculated from T1-weighted MRI data, (ii) metrics estimating structural connectivity, via tractography of white matter fibre bundles, calculated from diffusion-weighted data, and (iii) metrics estimating functional connectivity, via correlations analyses of the blood oxygen level dependent signals of distinct grey matter regions, calculated from resting-state functional MRI data. Concerning (2), the investigated study populations: The here-investigated study populations included not only (i) MRI data from patients with typical MND disorders such as ALS, but also with more uncommon MND subtypes such as ALS-FTD variants and PLS. Of particular notice is that for many patients, both ALS(-FTD) and PLS, (ii) longitudinal data were available, which were acquired across regular time periods (a typical inter-scan interval comprised four months), and up to six follow-up MR assessments were available for single patients. Finally (3), concerning the available rich clinical, genetic and neuropsychological characterisation of the study populations: Most patients were (i) clinically well-characterised, including careful recording of the major clinical MND assessment tools such as the ALSFRS_r score and the Penn Upper Motor Neuron Scale. Previous neurovascular, psychiatric and other neurological diagnoses were investigated to identify potential exclusion criteria; moreover, basic demographic and clinical data were recorded (including age, sex, handedness and years of education, mediations, body regions of symptoms onset and family history). (ii) Genetic profiling was available for most patients to identify genetic variants such as hexanucleotide repeat expansion in *C9orf72*. Finally, (iii) rich neuropsychological test batteries were collected for many patients, including assessment batteries to probe language functions or the Emotional Lability Questionnaire (ELQ), which were of particular importance for the studies of this thesis investigating extra-motor involvement. In summary, such standardised and multimodal MR data from a comparatively large, longitudinal, rare MND-subtypes-inclusive, clinically-/neuropsychologically-/genetically-well characterised MND cohorts has hardly been documented to be investigated using multimodal neuroimaging metrics before.

1.4 Main objectives

The main objective of the four peer-reviewed articles is to investigate multimodal neuroimaging metrics in MND and characterise how they relate to motor and extra-motor clinical involvement. More specifically, this was to be investigated multifold, including for, (i) common (ALS) and uncommon (PLS, ALS-FTD) MND disorders, (ii) cross-sectional and longitudinal assessments, as well as (iii) motor and extra-motor symptoms, including PBA and language deficits. In the following, the specific objectives for each article are provided separately in bullet points (the running titles are provided in brackets according to section 0).

- **Study 1 (“*Brainstem-cortex disconnection in ALS*”):**
 - Progressive cortico-medullary disconnection in ALS patients can be captured by multimodal neuroimaging metrics
 - Carriers of the genetic variant *C9orf72* exhibit higher ALS disease burden as compared to non-carriers
 - ALS patients without bulbar impairment show presymptomatic change, which can be captured using multimodal neuroimaging metrics
- **Study 2 (“*Language-associated brain regions in ALS-FTD*”):**
 - Detailed multimodal neuroimaging assessment of cerebral regions and networks which are physiologically linked to language domains in the ALS-FTD spectrum
 - Characterisation of specific phenotypes along the ALS-FTD spectrum using multimodal neuroimaging metrics
- **Study 3 (“*Motor connectome disintegration in PLS*”):**
 - Characterisation of progressive multimodal neuroimaging changes in PLS, focussing on exploring additional insights of rs-fMRI-based biomarkers and related advance neuroimaging metrics for routinely used clinical instruments
 - Comprehensive longitudinal analysis of motor network degeneration in PLS based on somatotopic segmentations and body region-based multimodal neuroimaging metrics
 - Investigation of pre- and supplementary motor area involvement in PLS and contrasting of sensitivity and practical utility of multimodal neuroimaging metrics
- **Study 4 (“*Pseudobulbar affect in PLS*”):**

- Detailed evaluation of multimodal neuroimaging correlates of PBA without anatomical *a priori* hypotheses in a large cohort of clinically- and genetically well-characterised cohort of primary lateral sclerosis patients
- Targeted assessment of the two most commonly proposed pathomechanisms of PBA using multimodal neuroimaging metrics, namely the contribution of corticobulbar and cerebellar disease-burden

1.5 Clinical relevance

The current study series provides evidence that multimodal neuroimaging sensitively captures motor and extra-motor involvement in MND. This sensitivity was demonstrated multifold, including for (i) common (ALS) and uncommon (PLS, ALS-FTD) MND disorders and (ii) cross-sectional and longitudinal assessments, as well as (iii) (i) motor and extra-motor symptoms, including language deficits and PBA. Our academic study results provide compelling evidence that multimodal neuroimaging metrics can further enhance future clinical trial protocols. Although such deficits have severe impacts on patients' quality of life and implications for caregivers, they are hardly taken into account as outcome measures in clinical trial protocols, probably because quantitative and standardized assessment has not yet been convincingly demonstrated. With our results, however, we believe to have provided such evidence and thereby hope to contribute to the integration of multimodal neuroimaging into the design of clinical trials, ultimately motivating the development of therapeutics specifically targeted at such severely the quality of life impairing symptoms in MND.

2 Chapter 1: “Brainstem-cortex disconnection in ALS”

Full title: Brainstem-cortex disconnection in amyotrophic lateral sclerosis: bulbar impairment, genotype associations, asymptomatic changes and biomarker opportunities

2.1 Introduction

Two of the key barriers to the development of effective disease-modifying therapies in amyotrophic lateral sclerosis (ALS) are the late inclusion into pharmacological trials, and lack of validated quantitative monitoring markers. Bulbar presentation in ALS has been consistently associated with shorter survival, faster functional decline and increased multidisciplinary support needs. Dysarthria has considerable quality of life implications and may impact on employment, social interactions and mood. Dysphagia may lead to weight loss, malnutrition, dehydration, aspiration pneumonia, sialorrhoea and increased risk for hospital admissions. Pseudobulbar affect may be misinterpreted as depression or behavioural change in the community, and may lead to social isolation. Despite these sombre sequelae, the substrate of bulbar impairment in ALS is relatively understudied radiologically, and proxies of bulbar impairment are also underrepresented among clinical trial outcome measures (1). Imaging studies in ALS overwhelmingly focus on cortical atrophy and corticospinal tract changes even though brainstem and corticobulbar tract degeneration are hallmark pathological features of ALS and have been associated with the condition since its earliest descriptions. In one of the first pathologically supported reports in 1867, Lockhart Clarke eloquently describes progressive bulbar involvement in ALS: “Her voice changed; she did not pronounce words as usual...Her deglutition now became difficult... The tongue is atrophied on each side, and in folds, reminding one of cerebral convolutions. Her talking is nearly unintelligible” (2). This moving description from over 150 years ago elegantly illustrates bulbar impairment in ALS which continues to affect patients today. Despite historical descriptions of brainstem atrophy and corticobulbar tract degeneration, these structures remain notoriously understudied in vivo. Brainstem pathology is regarded as ‘stage 1’ of a recently proposed four-stage pathological staging system based on pathological TDP-43 burden patterns (3), a staging-scheme increasingly supported by radiological data (4). Brainstem pathology is not unique to ALS, it is a shared feature of several motor neuron diseases (5), preferentially affecting the descending pyramidal tracts, cranial nerve nuclei or both. So, while

corticobulbar tract and brainstem pathology are ‘disease-defining’ features of ALS with dramatic clinical ramifications, they are seldom evaluated systematically from cortex to brainstem in large multimodal longitudinal imaging studies. Accordingly, the main objective of this study is the evaluation of a comprehensive panel of cortical, brainstem and cortex-brainstem connectivity metrics to appraise their longitudinal trajectory, discriminatory power and association with relevant clinical metrics. An additional objective is the characterisation of bulbar integrity and corticobulbar connectivity in patients carrying the GGGGCC hexanucleotide expansion in *C9orf72*. Moreover, as existing presymptomatic studies exclusively assess radiological changes in gene carriers, we specifically evaluate the radiological profile of sporadic patients with spinal onset disease who are asymptomatic from a bulbar perspective at the time of their scan to estimate bulbar and corticobulbar disease-burden prior to symptom manifestation. Our main hypothesis is that a panel of structural and functional MR metrics may capture progressive cortico-medullary disconnection. We also hypothesise larger disease burden in *C9orf72* carriers and some degree of presymptomatic change in patients without bulbar disability.

2.2 Methods

Participants

Imaging data from 198 patients with ALS and 108 healthy controls (HC) were included in this study (**Table 2.1**). All participants gave informed consent in accordance with the Ethics Approval of this research project (Beaumont Hospital, Dublin, Ireland – IRB REC08/90). A prospective study design was implemented with the recruitment of incidence ALS cases. The mean symptom duration of patients from symptom onset to first scan was 15.5 months (median 16) with a range of 5-27 months. Participating ALS patients were diagnosed according to the revised El Escorial criteria. Exclusion criteria included prior neurosurgery, prior cerebrovascular events, traumatic brain injury, comorbid neoplastic, or neuroinflammatory diagnoses. Patients with comorbid psychiatric disease, patients who were unable to tolerate MR scanning, patients with incomplete MR acquisition and patients without genetic information were excluded. Inter-scan interval for longitudinal follow-up was four months. In three main analysis streams, patients were either assessed as a single group (*analysis stream 1*), stratified by *C9orf72* status (*analysis stream 2*) or by the presence bulbar symptoms (*analysis stream 3*). Basic demographic and clinical variables (age, sex, handedness, years of education, medications, body region of symptom onset, family history of ALS or FTD), were recorded on the day of MRI scanning and all patients had their total ALSFRS-r and ALSFRS-r sub-scores documented at the time of their scan. All patients were screened for intronic GGGGCC repeat expansion in *C9orf72* by repeat-primed PCR. Capillary electrophoresis outcomes were visualised using GeneMapper version 4.0 and patients exhibiting 30 or more repeats were considered *C9orf72*-positive. Additionally, all participating patients were screened for a panel of protein-altering, exonic or splice-site variants present in 32 genes linked to ALS in the ALS online database (ALSod). (6)

MR imaging

MR data were acquired on a 3 Tesla Philips Achieva platform and the protocol included structural T1-weighted (T1w), resting-state functional MR (rsfMRI) and diffusion-weighted (DWI) pulse-sequences (**Figure 2.1**). The imaging protocol has been previously described (7). Briefly, T1-weighted (T1w) images were acquired with a 3D Inversion Recovery prepared Spoiled Gradient Recalled echo (IR-SPGR) sequence with the following parameters; field-of-view (FOV) of 256 x 256 x 160 mm, flip angle = 8°, spatial resolution of 1 mm³, SENSE factor = 1.5, TR/TE = 8.5/3.9 ms, TI = 1060 ms. Diffusion tensor images (DTI) were acquired with a spin-echo echo planar imaging (SE-EPI) pulse sequence using a 32-direction Stejskal-Tanner diffusion encoding scheme, FOV = 245 x

245 x 150 mm, 60 slices with no interslice gap, spatial resolution = 2.5 mm³, TR/TE = 7639 / 59 ms, SENSE factor = 2.5, b-values = 0, 1100 s/mm², dynamic stabilisation and spectral presaturation with inversion recovery (SPIR) fat suppression. Echo-planar imaging (EPI) was used to investigate fluctuations in the blood oxygen level-dependent (BOLD) signal for resting state functional imaging with eyes closed using the following imaging parameters: 30 axial slices, repetition time (TR) / echo time (TE) = 2000 ms / 35 ms, flip angle (FA) = 90°, pixel bandwidth = 1780, Hz/Px. Spatial resolution: 2.875 x 2.875 x 4 mm.

Cortical thickness measurements, medullary volume estimates and brainstem shape analyses

T1-weighted structural data were pre-processed in for cortical thickness (CT) calculations, medullary volume estimation, brainstem shape profiling and for downstream image registration. CT and medullary volumes were calculated using FreeSurfer version 7.1.0 (8) , including automated image segmentation, surface reconstruction and individual CT map output. We defined the bulbar segment of the motor cortex based on the *Brainnetome* atlas (9), which provides functional cortical parcellation based on multimodal imaging data. We refer to labels “A4tl_L” and “A4tl_R” (“tongue/larynx”) of the *Brainnetome* atlas as the “bulbar cortex”. For brainstem segmentation and subsequent medullary volume estimation, we relied on FreeSurfer’s *segmentBS* pipeline (10) , which uses a Bayesian algorithm to delineate probabilistic boundaries. To investigate brainstem outline deformations, the *first* pipeline (11) of the FMRIB’s Software Library was implemented (12); images were skull-stripped and bias-corrected using FSL’s *fsl_anat* pipeline. Subsequently, subcortical structures were segmented using a Bayesian approach and parametrized those labels as surface meshes to run statistical comparisons.

Appraisal of functional connectivity

Functional data were pre-processed to estimate functional connectivity (FC). FSL’s *feat* (13) was utilized for brain extraction, slice-time correction, and motion correction. Additionally, FSL’s *AROMA* algorithm (14) was implemented to correct for head-motion-related artifacts. Each patient’s pre-processed functional image was linearly co-registered to the native high-resolution structural scan using 6 degrees of freedom (DOFs), and for higher-level group comparisons, non-linearly warped to the MNI152 2mm standard space (12 DOFs). FC was defined as Fisher z-

Table 2.1 The demographic and clinical profile of study participants

	All ALS patients	HC	Welch two-sample t-test [W] or Chi-squared [C2] [ALS vs. HC]	C9+	C9–	BA (C9–)	BS (C9–)
Total number of subjects	198	108	<i>n.a.</i>	22	176	78	98
Age [y, mean $\pm$ SD]	59.78 $\pm$ 11.98	59.01 $\pm$ 10.72	W: $t(241.6) = -0.58$, $p = 0.57$	55.77 $\pm$ 8.45	60.29 $\pm$ 12.28	58.64 $\pm$ 12.84	61.60 $\pm$ 11.72
Sex, F/M	69/129	54/54	C2: $\chi^2(1, N = 306) = 6.06$, $p = 0.01^*$	9/13	60/116	23/55	37/61
Handedness, R/L	182/16	101/7	C2: $\chi^2(1, N = 306) = 0.08$, $p = 0.78$	19/3	163/13	72/6	91/7
Years of education [y, mean $\pm$ SD]	13.56 $\pm$ 3.45	14.69 $\pm$ 3.55	W: $t(215.07) = 2.67$, $p = 0.008^*$	13.73 $\pm$ 3.47	13.54 $\pm$ 3.46	13.55 $\pm$ 3.42	13.53 $\pm$ 3.51
ALSFRS-r score $\pm$ SD (baseline)	37.97 $\pm$ 7.18	<i>n.a.</i>	<i>n.a.</i>	31.68 $\pm$ 11.77	38.76 $\pm$ 5.98	40.68 $\pm$ 4.88	37.22 $\pm$ 6.35
Baseline scans [count]	198	108	<i>n.a.</i>	22	176	78	98
4-month follow-up [count]	107	18	<i>n.a.</i>	7	100	48	52
8-month follow-up [count]	65	13	<i>n.a.</i>	7	58	26	32
12-month follow-up [count]	34	8	<i>n.a.</i>	3	31	12	19
16-month follow-up [count]	2	0	<i>n.a.</i>	0	2	2	0

ALS: amyotrophic lateral sclerosis, ALSFRS-r: revised ALS functional rating scale, BA: bulbar asymptomatic, defined as *C9orf72* negative with spinal onset disease and no bulbar symptom at baseline, BS: bulbar symptomatic, defined as *C9orf72* negative patient with bulbar symptoms at baseline, C9+: hexanucleotide repeat expansion carrier in *C9orf72*, C9–: tested negative for hexanucleotide repeat expansions in *C9orf72*, F: female, HC: healthy control, L: left-handed, M: male, MRI: magnetic resonance imaging, N: sample size, *n.a.*: not applicable, R: right-handed, SD: standard deviation, y: years, *: significant at an alpha-level of $p \leq 0.05$

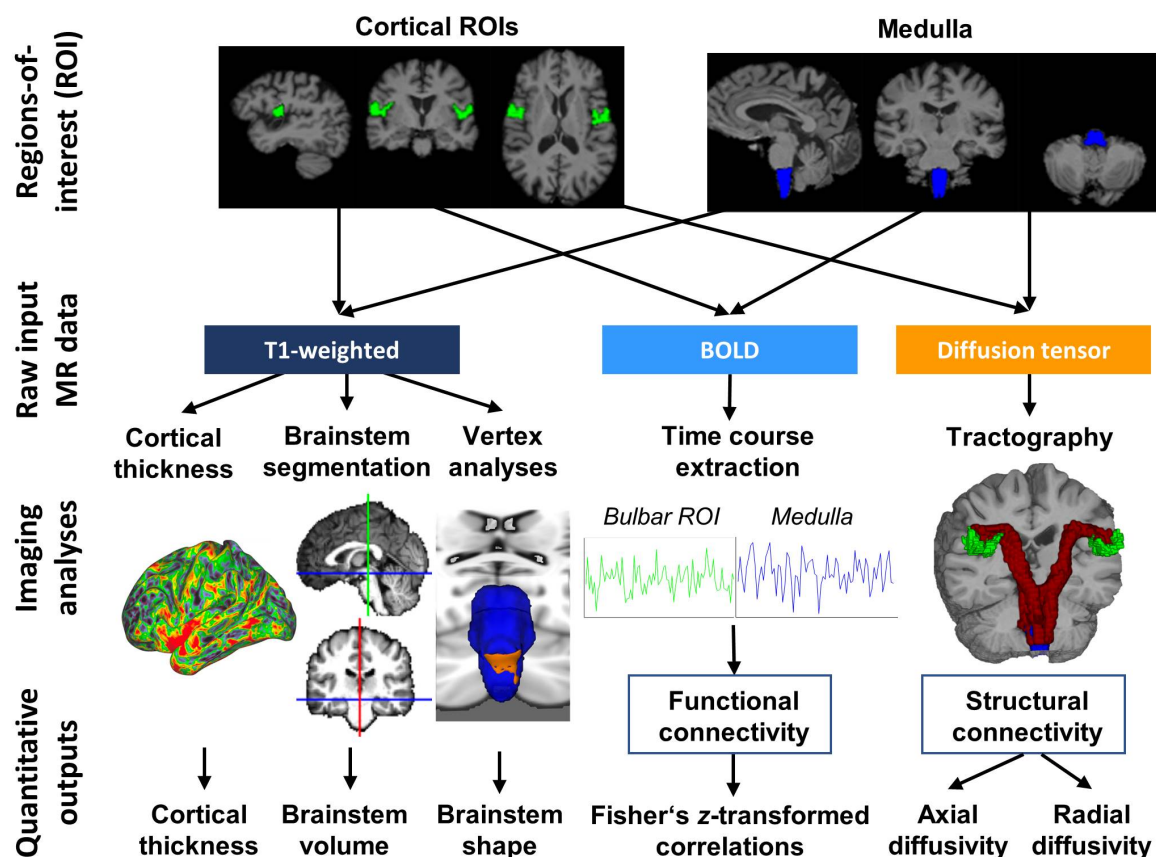


Figure 2.1 The workflow of data analyses from raw data to anatomical integrity metrics. A panel of cortical, brainstem, structural and functional cortico-medullary imaging measures were evaluated in a prospective, single-centre, longitudinal study. AD: axial diffusivity, ALS: patients with amyotrophic lateral sclerosis, BOLD: blood-oxygen-level-dependent signal, CT: cortical thickness, DTI: diffusion tensor imaging, DWI: Diffusion-weighted imaging, FC: functional connectivity, fMRI: functional MRI, HC: Healthy control, RD: radial diffusivity, ROI: Region of interest

transformed correlation between the mean time courses of the brainstem and the bulbar cortex (separately for the two hemispheres). FC was calculated within *Matlab R2021b* using tools from the CoSMoMVPA toolbox (15).

Retrieval of structural connectivity metrics

To determine structural connectivity (SC), diffusion-weighted (DW) data were used. Tools from *MRtrix3* (version 3.0.3) (16) were used for pre-processing including noise removal (17), removal of Gibb's Ringing artifacts (18), motion / eddy current (19) and bias-field corrections (20). Pre-processed DW images were then aligned to high-resolution T1-weighted data. As fractional anisotropy (FA) is a composite metric of the three eigenvalues (λ_1 , λ_2 , λ_3), it is histologically non-

specific to the underlying white matter pathology and merely offers an overall proxy of white matter integrity. Accordingly, in our study a diffusion-tensor model was fitted to the data to estimate cortico-medullary axial diffusivity (AD) (λ_1) and radial diffusivity (RD) $((\lambda_2 + \lambda_3)/2)$ separately (21). Tractograms were calculated between the medulla and the right/left bulbar cortices separately in each participant in native space. A probabilistic algorithm was implemented to produce a fixed number of streamlines ($n = 5000$) between the pairwise ROIs. We then binarized these tractograms to extract the mean AD/RD values per tract in each subject using the previously calculated tensor maps. Multimodal MR data of patients and controls were interrogated both for baseline differences (cross-sectional modelling) as well as differences in progression (longitudinal modelling). Not only disease-associated signatures were explored (*analysis stream 1*) by contrasting all ALS patients to controls, but two specific ALS cohorts were further evaluated. The imaging profile of hexanucleotide repeat expansion carriers in *C9orf72* (“C9+”) (*analysis stream 2*) and bulbar asymptomatic (BA) patients (*analysis stream 3*) i.e. C9-, spinal onset patients with a maximum ALSFRS-r bulbar score, were evaluated in dedicated analyses.

Statistical modelling

The age and education profile of patients and controls were contrasted using Welch two-sample t-tests and the ratios of gender and handedness distributions examined by Chi-square testing. All statistical analyses were performed within RStudio (R version 4.2.1) (22). Multimodal MR data of patients and controls were interrogated both for baseline differences (cross-sectional modelling) as well as differences in progression (longitudinal modelling). Not only disease-associated signatures were explored (*analysis stream 1*) by contrasting all ALS patients to controls, but two specific ALS cohorts were further evaluated. The imaging profile of hexanucleotide repeat expansion carriers in *C9orf72* (“C9+”) (*analysis stream 2*) and sporadic bulbar asymptomatic (BA) patients (*analysis stream 3*) i.e. C9-, spinal onset patients with a maximum ALSFRS-r bulbar score, were evaluated in dedicated analyses. Each of these streams were explored cross-sectionally and longitudinal, whereby the cross-sectional model comprised a simple linear model for *analysis stream 1* and a one-way analysis of variance (ANOVA) for *analysis streams 2* and *3* (given that these contrasted three groups). Age, sex and handedness were included as covariates and volumetric analyses were also adjusted for total intracranial volume (TIV). Additionally, post-hoc Tukey’s Honest Significance Difference (HSD) testing was implemented to explore pairwise differences between groups. To evaluate brainstem outline alterations at baseline, non-parametric statistical comparisons were implemented between all ALS patients and HC (*analysis*

stream 1), C9+ vs. C9- ALS patients (analysis stream 2) and BA vs. bulbar symptomatic (BS) C9- patients (analysis stream 3). For each comparison, we applied FSL's randomise (23) algorithm with 5000 permutations and 2D-optimized threshold-free cluster enhancement (TFCE), using a two-sample t-test design covarying for age, gender and handedness. Since two-sided testing was performed, significance threshold was set to $p \leq 0.025$. Longitudinal changes were evaluated in linear mixed effects models, where Time (i.e. imaging timepoint) was considered as a random effect and the subjects as fixed effects. In “analysis stream 1”, the interaction between Time and Group, in “analysis stream 2” progression differences between C9+ vs. C9-/HC and in “analysis stream 3”, differences between C9- BA and BS patients were evaluated. Similar to the cross-sectional analyses at baseline, age, sex and handedness were included as covariates. To test the discriminatory potential of specific imaging metrics in our panel of radiological indices, we ran Receiver Operator Characteristics (ROC) analyses between all ALS patients and controls and tested the likelihood that a given area under the curve (AUC) value differed from 0.5. Given the relentless clinical progression in ALS, we hypothesized that AUCs may increase over time, therefore we included only patients who had a baseline and a 12-month follow-up scan (N = 67 vs. 147 HC) and computed ROC and corresponding AUC values for each imaging metrics at the two time-points 12-months apart. To test the hypothesis of increasing AUCs, we ran a one-sided paired t-test between the AUC of all 9 analysed imaging metrics at baseline and follow-up. The ROC/AUC analyses were carried out within RStudio using the pROC package (24). Clinico-radiological associations were explored for bulbar motor (ALSFRS-r) scores. Cross-sectional associations with bulbar subscores at baseline were tested in a linear model incorporating the relevant covariates. Longitudinal associations were investigated based on the change in ALSFRS-r bulbar subscores as the independent, and the difference between the given MRI metric as dependent variables, correcting for age, gender and handedness.

2.3 Results

Demographics

Demographic and clinical data are presented in **Table 2.1**. Welch two-sample *t*-test revealed no significant age differences between ALS patients and controls ($t(241.6) = -0.58, p = 0.57$). Chi-square test captured sex differences ($\chi^2(1, N = 306) = 6.06, p = 0.01$), but no differences in handedness ($\chi^2(1, N = 306) = 0.08, p = 0.78$). All patients tested negative for a panel of protein-altering, exonic or splice-site variants in 32 genes linked to ALS.

Progressive cortico-medullary disconnection is specific for ALS

The main statistical outputs of neuroimaging analyses are presented in **Table 2.2** and illustrated in **Figures 2.2–2.5**. Considering all ALS patients (**Figure 2.2**), more rapid cortico-medullary disconnection was identified in patients compared to controls in the right hemisphere. The interaction effects (*Group x Time*) in the longitudinal models revealed RD increase [$t(237) = 2.030, p = 0.044$], AD increase [$t(237) = 2.210, p = 0.028$] and FC decline over time [$t(237) = -2.187, p = 0.030$]. Interestingly, the CT of the bilateral bulbar cortex – while not exhibiting progressive change – was significantly thinner in patients at baseline (both RH/LH: $p < 0.001$). No medullary volume (MV) or brainstem shape (BrS) differences were detected between the study groups.

The discriminatory profile of bulbar neuroimaging metrics and their longitudinal trajectory

Our ROC analyses and the derived AUC values confirmed the discriminatory potential of nearly all analysed neuroimaging metrics. At baseline, medullary volume showed the highest discriminatory power for distinguishing patients from controls (AUC = 0.611, $p = 0.006$, **Figure 2.3A**), followed by CT (LH: AUC = 0.619, RH: AUC = 0.567) and RD (LH: AUC = 0.606, RH: AUC = 0.606), all exhibiting high AUCs. Notably, the AUCs of all analysed metrics increased over time ($t(8) = 5.431, p < .001$; **Figure 2.3B**).

Cortical thickness decreases more rapidly in hexanucleotide repeat expansion carriers

Following patient stratification by *C9orf72* status (**Figure 2.4**), CT was identified as an important moderator of progression: bulbar cortex CT loss in C9+ patients was more rapid than in C9- or HC group [RH: $t(236) = -2.040, p = 0.042$; LH: $t(236) = -3.505, p < 0.001$; based on the *Group x Time* interaction effect with C9+ as a reference group]. Moreover, ANOVA confirmed significant bilateral CT differences already at baseline [RH: $F(2,298) = 14.11, p < 0.001$; LH: $F(2,298) = 2.040, p = 0.052$], whereas post-hoc Tukey HSD testing revealed that C9+ patients

Table 2.2 Cross-sectional and longitudinal outputs for the panel of imaging metrics

	Cross-sectional (Main effect: Group)						Longitudinal (Interaction: Time x Group)					
	Right hemisphere			Left hemisphere			Right hemisphere			Left hemisphere		
	Estimate	t / F-value	p-value	estimate	t / F-value	p-value	estimate	t / F-value	p-value	estimate	t-value	p-value
ALS vs. HC												
Vol	-6.75e-5	-1.505	0.133	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	-3.20e-5	-1.263	0.208	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
CT	-0.076	-3.375	8.34e-4*	-0.088	-4.315	<.001*	-0.022	-1.686	0.093	-6.20e-3	-0.639	0.524
RD	-2.26e-5	-1.376	0.170	-7.63e-6	-0.454	0.651	2.58e-5	2.030	0.044*	2.07e-5	1.626	0.105
AD	-7.58e-5	-2.151	0.032*	-4.74e-5	-1.320	0.188	6.01e-5	2.210	0.028*	4.35e-5	1.567	0.118
FC	0.026	1.035	0.302	0.005	0.185	0.853	-0.048	-2.187	0.030*	0.004	0.174	0.862
C9+ vs. C9-/HC												
Vol	9.40e-7	3.427	0.034*	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	5.82e-6	0.161	0.872	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
CT	0.920	14.11	<.001*	0.709	12.85	<.001*	-0.029	-2.040	0.042*	-0.044	-3.505	<.001*
RD	9.30e-8	2.533	0.081+	4.40e-8	1.144	0.320	-1.56e-5	-0.746	0.456	2.04e-8	9.75e-4	0.999
AD	5.57e-7	3.276	0.039*	3.17e-7	1.794	0.168	-2.72e-5	-0.597	0.551	-1.50e-5	-0.325	0.745
FC	0.050	0.599	0.550	0.006	0.079	0.924	-0.003	-0.074	0.941	-0.024	-0.651	0.515
BA/BS vs. HC (C9- patients only)												
Vol	1.03e-6	3.813	0.023*	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	3.80e-5	1.543	0.124	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
CT	0.300	4.707	0.010*	0.433	8.233	<.001*	0.0172	1.654	0.100+	1.03e-4	0.011	0.991
RD	1.65e-7	4.460	0.012*	1.18e-7	3.042	0.049*	-3.11e-5	-2.183	0.030*	-3.97e-6	-0.318	0.095+
AD	1.03e-6	6.074	0.003*	8.01e-7	4.500	0.012*	-6.96e-5	-2.304	0.022*	-5.45e-5	-1.778	0.077+
FC	0.076	0.905	0.406	0.043	0.507	0.603	0.048	1.961	0.051+	-0.021	-0.862	0.390
MRI-clinical correlations (ALSFRS-r bulbar score)												
Vol	266.22	0.361	0.7193	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	3.59e-05	0.809	0.421	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
CT	-0.638	-0.565	0.574	-0.567	0.508	0.613	-5.12e-4	-0.027	0.978	-0.012	-0.949	0.346
RD	1.58e3	0.868	0.389	8.18e2	0.454	0.651	-3.14e-5	-1.286	0.202	-1.58e-5	-0.578	0.565

AD	689.01	0.808	0.422	711.43	0.843	0.402	-5.17e-5	-0.974	0.333	-3.10e-5	-0.541	0.590
FC	1.032	0.883	0.380	-0.324	-0.251	0.802	9.88e-4	0.022	0.982	5.57e-3	-0.119	0.906
Receiver operating characteristics (ROC)												
ROI							AUC at baseline			AUC at 12 months		
Medulla volume							0.611			0.642		
CT right							0.567			0.624		
CT left							0.619			0.685		
RD right							0.606			0.670		
RD left							0.606			0.682		
AD right							0.563			0.645		
AD left							0.467			0.612		
FC left							0.504			0.535		
FC right							0.521			0.553		

For unilateral structures, such as the medulla, relevant values are presented in the “left hemisphere” columns. For the cross-sectional comparisons “ALS vs. HC” and the “MRI-clinical correlations”, *t*-tests are reported; for the cross-sectional comparisons “C9+ vs. C9/HC” and “BA/BS vs. HC” we *F*-tests (analyses of variance) are reported. Where the *F*-test was significant for cross-sectional comparisons, we performed pairwise post-hoc tests. Significant post-hoc differences are indicated in figures 4 and 5 using asterisks. AD: axial diffusivity, ALS: amyotrophic lateral sclerosis, AUC: area under the curve, BA: bulbar asymptomatic, defined as C9orf72 negative with spinal onset disease and no bulbar symptom at baseline, BS: bulbar symptomatic, defined as C9orf72 negative patient with bulbar symptoms at baseline, C9+: hexanucleotide repeat expansion carrier in C9orf72, C9-: tested negative for hexanucleotide repeat expansions in C9orf72, CT: cortical thickness, FC: functional connectivity, HC: healthy control, MRI: magnetic resonance imaging, n.a.: not applicable, RD: radial diffusivity, ROC: receiver operating characteristics, Vol: medullary volume, + approaching significance at an alpha-level of $p \leq 0.10$, * significant at an alpha-level of $p \leq 0.05$

were the main drivers of this effect [RH: thinner CT of C9+ vs. C9-: $p < 0.001$; LH: thinner CT of C9+ vs. C9-: $p < 0.001$]. The comparison of AD also revealed a genotype-effect [RH: $F(2,298) = 14.11$, $p = 0.0391$], but pairwise group differences could not be confirmed by post-hoc Tukey HSD testing. Data visualization (**Figure 2.4C**) reveals a tendency for increased AD in both ALS groups vs. HC. Brainstem volumes and brainstem shape profiles were not modulated by genetic status.

Bulbar asymptomatic patients exhibit multi-level presymptomatic alterations

Spinal onset C9- patients without bulbar manifestations (“bulbar asymptomatic”- BA) exhibit marked radiological changes at baseline (**Figure 2.5**) based on four neuroimaging metrics:

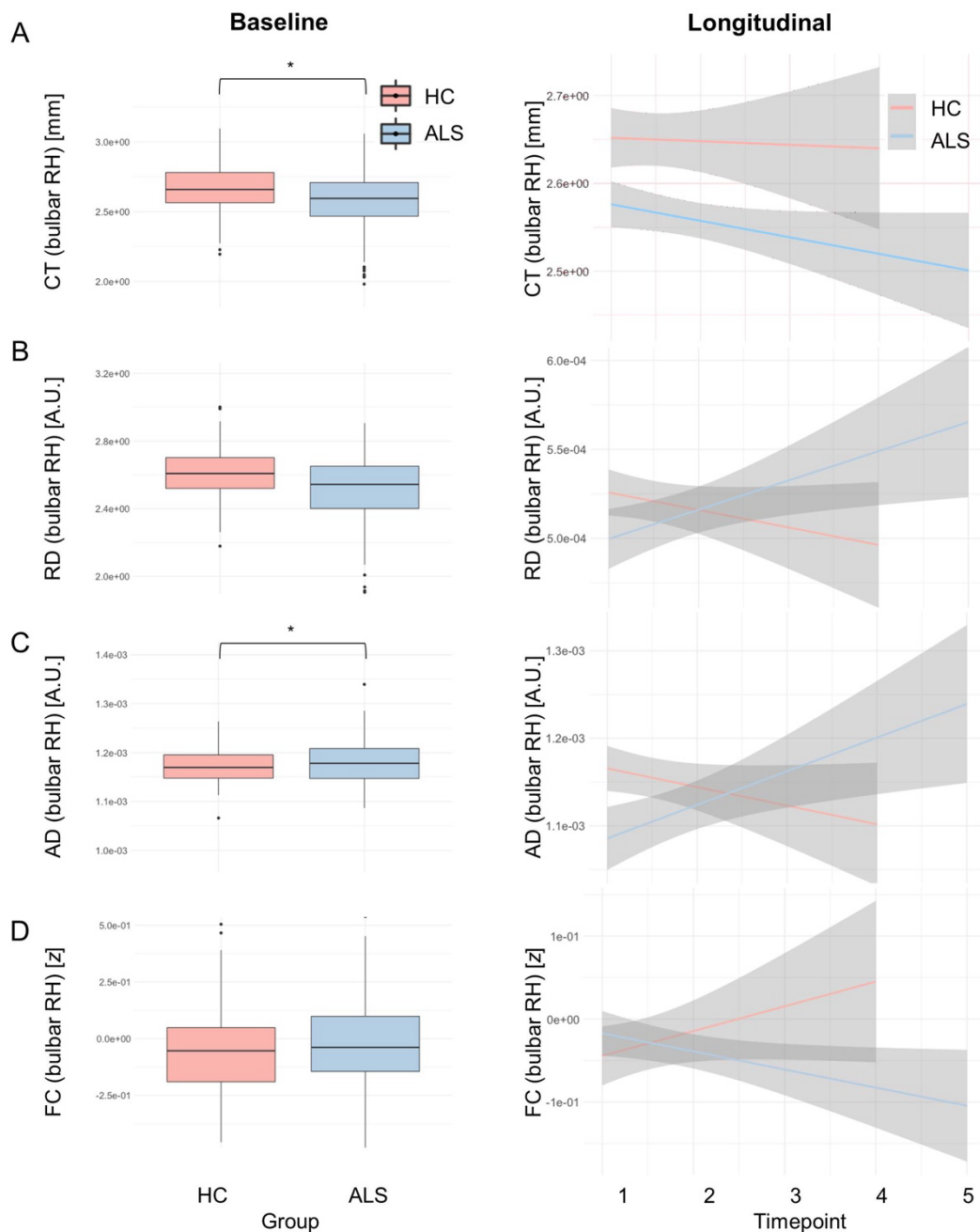


Figure 2.2 Analysis stream 1: Disease-associated imaging traits in unselected (all) ALS patients. Cross-sectional (left panel) and longitudinal (right panel) imaging alterations in unstratified ALS patients with reference to healthy controls. (A) Cortical thickness of the right bulbar cortex and (C) cortico-medullary axial diffusivity differentiates ALS from controls at baseline. Connectivity metrics reveal progressive cortex-brainstem disconnection based on (B) radial diffusivity (RD), (C) axial diffusivity (AD) and (D) functional connectivity (FC). Detailed descriptive statistics are presented in Table 2. Boxplots represent medians ± 1 quartile, whiskers denote a data range spanning the median $\pm 1.58 \times IQR\sqrt{n}$, dots represent outliers, whereby IQR is the interquartile range. * denotes statistical significance of unpaired t-tests, thresholded at $p \leq 0.05$. AD: axial diffusivity, ALS: patients with amyotrophic lateral sclerosis, CT: cortical thickness, FC: functional connectivity, HC: Healthy control, IQR: interquartile range, RD: radial diffusivity

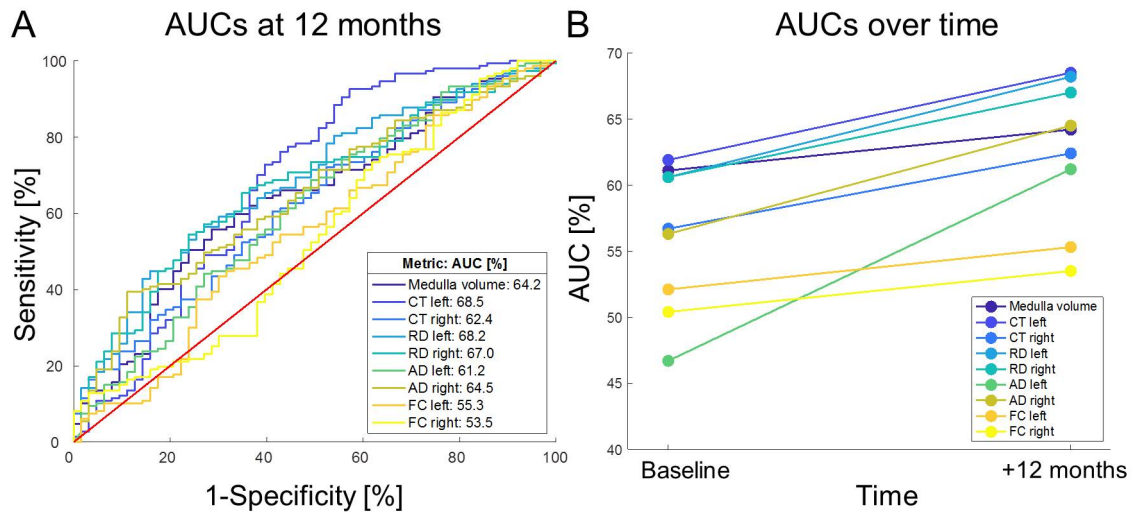


Figure 2.3 The discriminatory hierarchy of bulbar neuroimaging markers between patients with ALS and healthy controls based on area under the curve (AUC) values their Receiver Operator Characteristics (ROC) analyses (A). AUC values of all bulbar metrics increased over a 1-year follow-up (B) ($p < 0.001$).

Figure 2.5

(1) medullary volume [$F(2,276) = 1.03e-6$, $p = 0.023$]; (2) bilateral CT [RH: $F(2, 276) = 0.300$, $p = 0.010$; LH: $F(2, 276) = 0.433$, $p < 0.001$], (3) bilateral cortico-medullary RD [RH: $F(2, 276) = 1.65e-7$, $p = 0.012$; LH: $F(2, 276) = 1.18e-7$, $p = 0.049$] and (4) bilateral cortico-medullary AD [RH: $F(2, 276) = 1.03e-6$, $p = 0.003$; LH: $F(2, 276) = 8.01e-7$, $p = 0.012$]. Post-hoc Tukey HSD tests confirmed left-hemispheric CT reduction even in bulbar asymptomatic patients compared to controls ($p = 0.017$). From a cortico-medullary connectivity perspective, BA patients did not differ from BS patients in right-hemispheric RD ($p = 0.061$) and with regards to left-hemispheric RD, BA patients exhibited higher values compared to BS ($p = 0.044$). No post-hoc differences were detected in medullary volumes or FC in pairwise comparisons. Longitudinally, right-hemispheric structural connectivity – both RD and AD – deteriorated in BA similarly to BS with reference to controls [RD: $t(219) = -2.183$, $p = 0.030$; AD: $t(219) = -2.304$, $p = 0.022$. Moreover, right-hemispheric FC tended to decrease in BA similarly to BS vs. controls [FC: $t(219) = 1.961$, $p = 0.051$]. These observations suggest that considerable disease burden can be ascertained in anatomical regions associated with bulbar function before bulbar disability develops, both at a cortical level and also from a cortico-medullary connectivity perspective. No presymptomatic shape alterations were detected in the BA group.

Dissociation between bulbar disability scores and imaging metrics

No direct correlations were identified between bulbar ALSFRS-r subscores and any of the radiological integrity metrics (CT, AD, RD, FC, MV, BrS), neither cross-sectionally nor longitudinally. Output statistics are summarized for all neuroimaging metrics in **Table 2.2** to demonstrate the dissociation between motor disability and cerebral imaging measures.

2.4 Discussion

We have evaluated the integrity of anatomical structures involved in bulbar function in a large cohort of genetically and clinically characterised patients in a longitudinal imaging study using a standardised imaging protocol. Our analyses revealed progressive structural and functional disconnection between the motor cortex and the brainstem over time. Cortical thickness reduction was an early feature on cross-sectional analyses with limited further progression on longitudinal follow-up. Hexanucleotide repeat carriers exhibited lower brainstem volumes, lower cortico-medullary structural connectivity and faster cortical thinning. While brainstem and corticobulbar tract involvement are well established post mortem, these brain regions are challenging to quantitatively evaluate in vivo. Despite its considerable clinical implications, the substrate of bulbar impairment in ALS is poorly characterised at present. ALS is associated with progressive brainstem-cortex disconnection which is particularly rapid in *C9orf72* hexanucleotide repeat carriers. The systematic analysis of a large panel of imaging metrics demonstrates that some metrics show discriminatory potential between patients and controls at baseline, but exhibit limited change over time; these may be ideally suited for diagnostic applications. Conversely, other metrics may not readily discriminate patients from controls at baseline, but capture subtle changes over very short follow-up periods, making them particularly useful for monitoring applications. Our study also highlights that despite preserved bulbar function at the time of MR imaging, significant degenerative change can already be observed in the relevant brain regions. Disease burden in ALS is best evaluated in by robust multimodal studies and academic studies have the potential to inform the design of streamlined pharmacological trial protocols. The combination of a fast-acquisition 3D T1-weighted and a diffusion tensor imaging protocol offer ample biomarker potential both for clinical trial applications obviating the need for complex fMRI analyses.

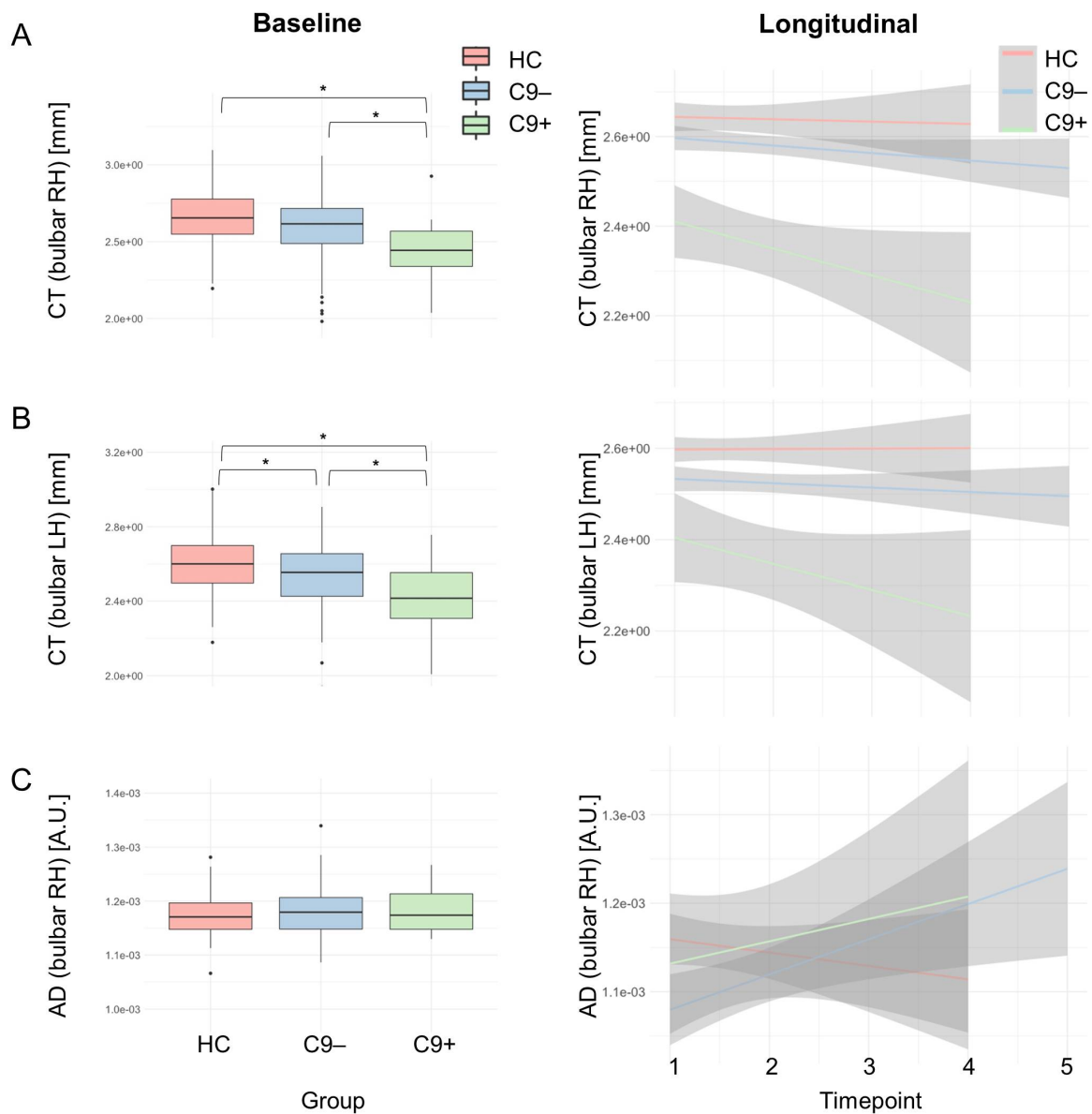


Figure 2.4 Analysis stream 2: the evaluation of bulbar imaging indices in GGGGCC hexanucleotide repeat expansion carriers in *C9orf72*. Cross-sectional patterns (left panel) and longitudinal changes (right panel) were evaluated at cortical level, in cortico-medullary connectivity and in the brainstem. Detailed descriptive statistics are presented in Table 2. Boxplots represent medians $\pm$ 1 quartile, whiskers denote a data range spanning the median $\pm 1.58 \times IQR\sqrt{n}$, dots represent outliers, whereby IQR is the interquartile range. * denotes statistical significance of unpaired t-tests, thresholded at $p \leq 0.05$. AD: axial diffusivity, ALS: patients with amyotrophic lateral sclerosis, C9+: ALS patients with GGGGCC hexanucleotide repeat expansion in *C9orf72*, C9-: ALS patients without GGGGCC hexanucleotide repeat expansion in *C9orf72*, CT: cortical thickness, FC: functional connectivity, HC: Healthy control, IQR: interquartile range, RD: radial diffusivity

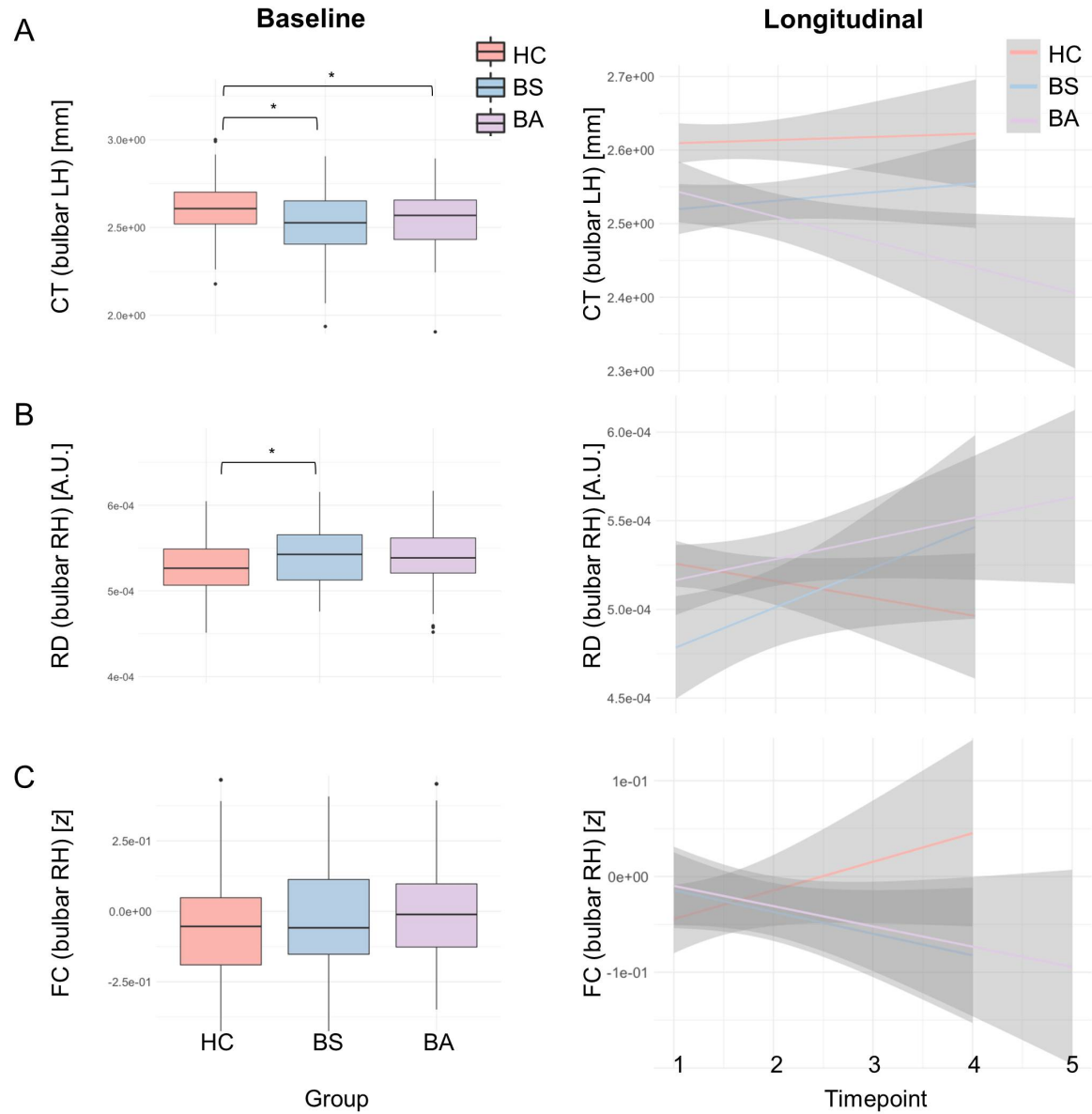


Figure 2.5 Analysis stream 3: the evaluation of “bulbar asymptomatic” patients i.e. sporadic patients with ALS with spinal onset disease without bulbar symptoms, without a family history of ALS, who tested negative for *C9orf72*. Cross-sectional patterns (left panel) and longitudinal changes (right panel) were evaluated at cortical level, in cortico-medullary connectivity and in the brainstem. Patients who are asymptomatic from a bulbar disability perspective, already exhibit cortical thickness reductions at baseline. Detailed descriptive statistics are presented in **Table 2.2**. Boxplots represent medians ± 1 quartile, whiskers denote a data range spanning the median $\pm 1.58 \times IQR\sqrt{n}$, dots represent outliers, whereby IQR is the interquartile range. * denotes statistical significance of unpaired t-tests, thresholded at $p \leq 0.05$. AD: axial diffusivity, ALS: patients with amyotrophic lateral sclerosis, BA: “bulbar asymptomatic” C9- patients without bulbar symptoms BS: C9- patients with bulbar symptoms, C9-: ALS patients without GGGGCC hexanucleotide repeat expansion in *C9orf72*, CT: cortical thickness, FC: functional connectivity, HC: Healthy control, IQR: interquartile range, RD: radial diffusivity

The targeted evaluation of a cohort of hexanucleotide expansion carriers in *C9orf72* confirmed the unique clinical and radiological attributes of this genotype. The radiological signature of *C9orf72* is classically associated with marked frontotemporal change and resulting cognitive dysfunction. It is increasingly clear however that marked frontotemporal change in ALS is not unique to *C9orf72* (25) and that patients with this genotype may also have distinguishing cerebellar, spinal cord and other extra-motor changes (7, 26). Our finding of a more rapid neurodegenerative process in this cohort is well in line with both clinical observations and other neuroradiology studies (27).

Presymptomatic changes are of huge interest in ALS and considerable pathological change has been consistently demonstrated in mutation carriers (26, 28, 29). Pioneering studies of presymptomatic disease-burden not only offer a window on incipient changes, the sequential involvement of anatomical structures and propagation patterns for academic research (4, 30), but from a pragmatic, clinical view point, they may inform the ideal timing of future pharmacological interventions (31, 32). Familial cases and carriers of pathogenic mutations however only represent a small minority of patients with ALS. It is likely that “sporadic” patients also accrue disease burden long before symptom onset and certainly well before the diagnosis is confirmed. Radiological observations from presymptomatic *SOD1* and *C9orf72*, while conceptually important, may not be directly transferrable to “sporadic” ALS due to their distinctive anatomical signatures and differing progression rates. Accordingly, the presymptomatic phase of “sporadic ALS” remains notoriously elusive and we currently merely rely on indirect insights derived from gene carriers. Recent presymptomatic studies describe slowly progressive neurodegenerative changes decades before symptom onset, and raise the possibility of developmental factors (33). It has been speculated that ample degenerative change has to take place for symptom manifestation, and that there may be a certain threshold when compensatory circuits and inherent functional redundancy are exploited. Analogous to the concept of cognitive reserve, terms such as “motor reserve” have been coined (34), but not compellingly demonstrated. As we have shown in this study, it may be possible to study symptomatic cohorts of patients who are asymptomatic in a specific clinical domain, in our case bulbar function, and appraise the integrity of the relevant structures involved in that specific function. Our study indicates, that despite preserved bulbar function in sporadic patients with ALS, significant degenerative change can already be observed in relevant brains regions.

One of the many roles of academic neuroimaging studies is to critically appraise the practical utility of a spectrum of radiology metrics to inform the design of streamlined clinical trial applications. While MRS, QSM, rsfMRI, NODDI, spinal cord metrics, etc. all offer invaluable academic insights (26, 35-41), they are not routinely implemented in the clinical setting. As demonstrated by this study, a high-resolution structural dataset can be flexibly interrogated in a multitude of pipelines and a multitude of open-source software libraries are available for transparent data interpretation. Similar to the versatility of structural data, DWI/DTI data can be meaningfully interrogated by tractography, tract-based statistical approaches or in connectomic models (42-44). Our data indicate that contrary to previous reports (45), vertex analyses have relatively little to offer at a brainstem level; brainstem outline alterations merely reflect overall shape deformations and may not meaningfully capture focal pathology in relevant structures such as cranial nerve nuclei or descending corticospinal tract degeneration. Similarly, the assessment of medullary volumes revealed no disease-associated or genotype-specific signatures either cross-sectionally or longitudinally. The absence of medullary volume reduction is not surprising given the selective and focal involvement of specific brainstem structures instead of a more global process. Another practical aspect of protocol development is ease of data harmonisation (46, 47) which is particularly pertinent to low-incidence conditions such as ALS requiring multi-site collaborations for sufficient statistical power. Clinical trials are also invariably multi-site, necessitating stringent protocol harmonisation. In our study, the discriminatory potential of bulbar imaging measures between patients and controls were evaluated by receiver operating characteristic analyses. While AUC values did not reach 0.7 which is commonly regarded as a cut-off for excellent discrimination, at 12-month follow-up, most of the AUC values were over 0.6 suggestive of acceptable discrimination. Machine-learning frameworks have been increasingly applied to large ALS datasets (48, 49) and feature importance analyses have invariably highlighted the role of cortical grey and white matter diffusivity measures (36, 50-55). To demonstrate the diagnostic utility of such models however, classification models need to be tested and validated on early-stage patients or patients soon after their diagnoses (56). The accurate categorisation of late-stage or patients with considerable disability says relatively little about the practical utility of a particular model. This notion is demonstrated by the AUC profile of our panel of bulbar metrics which all increase over time (**Figure 2.1**).

Patients with pseudobulbar affect (PBA) experience sudden tearing or laughing in response to minimal emotional stimuli. Patients with PBA are well aware of their exaggerated reactions and

often choose to avoid social interactions (57). In recognition of the considerable quality of life implications of PBA, a multitude of pharmacological trials have been conducted recently (58). While the classical conceptualisation of pseudobulbar affect centres on the loss of corticobulbar inhibition i.e. cortico-medullary disconnection, more recent PBA studies highlight the role of impaired cerebellar gating as well as extra-motor control network dysfunction (57, 59-63). We also note that an interaction between cognitive manifestations and bulbar impairment has been consistently suggested by epidemiology, neuroimaging and neuropsychology studies (30, 64-67) and the more detailed assessment of descending frontopontine, temporopontine and parietopontine fibres may reveal additional insights. Finally, it is noteworthy that corticobulbar tract degeneration and bulbar dysfunction are not unique to ALS, but also commonly observed in primary lateral sclerosis (PLS) (68-70). While PLS typically manifest with lower limb spasticity initially, spastic dysarthria and pseudobulbar affect commonly ensue over the course of the disease (71, 72). The development of non-invasive cortico-medullary connectivity measures may therefore be relevant to other neurodegenerative conditions, and more broadly, to other conditions where pseudobulbar dysfunction is an important feature, such as multiple sclerosis (MS).

This study is not without limitations. The systematic evaluation of bulbar lower motor neuron involvement was not possible due to the lack of availability of standardised EMG measures to the authors. We acknowledge the scarce follow-up data on the healthy control cohort, which were acquired to account for healthy ageing, but more complete normative data sets would be desirable for accurate longitudinal modelling. Moreover, the lack of post mortem data precludes the histopathological validation of our radiological findings. Notwithstanding these limitations, our data demonstrates progressive cortex-brainstem disconnection as a unifying feature of ALS biology.

2.5 Conclusions

ALS is associated with progressive brainstem-cortex disconnection which is particularly rapid in *C9orf72* hexanucleotide repeat carriers. There are no direct associations between bulbar disability and cerebral imaging metrics, suggesting that bulbar symptoms may be predominantly driven by lower motor neuron dysfunction. Imaging indices differ considerably in their detection sensitivity and ability to track progressive pathological changes. Disease burden in ALS is therefore best evaluated by large panels of complementary imaging markers. Academic studies have the potential to inform the design of streamlined clinical and future pharmacological trial protocols.

3 Chapter 2: “Language-associated brain regions in ALS-FTD”

Full title: The involvement of language-associated networks, tracts and cortical regions along the ALS-FTD spectrum: a structural-functional computational neuroimaging study

3.1 Introduction

The clinical profile of ALS is classically associated with bulbar dysfunction, respiratory weakness, decline in dexterity and gait impairment (1). A substantial body of evidence from neuropsychology, neuroimaging, and post mortem studies have demonstrated varying degree of frontotemporal involvement (67, 73-75) and the clinical overlap between ALS and FTD has been cemented by the discovery of shared genetic variants such as hexanucleotide repeat expansion in *C9orf72* (76). The spectrum of neuropsychological manifestations in ALS are often thought to be dominated by executive dysfunction and behavioural impairment, but the high prevalence of memory impairment, apathy, deficits in social cognition and language impairment are increasingly recognised, further supporting the concept of an ALS-FTD spectrum (77). A number of staging approaches have been proposed to stratify patients with ALS based on cognitive, behavioural, disability, pathological and imaging criteria (3, 47, 64, 78-80) recognising the fundamental heterogeneity of the disease and helping to designate smaller, more homogenous subgroups.

Language impairment in ALS is increasingly recognised (81). It has considerable quality of life ramifications and may impact on employment, understanding information, enunciating care preferences, social interactions, engagement with support providers. Language impairment may lead to social withdrawal, low mood, depression and may potentially impact on participation in clinical trials. While numerous well-validated instruments exist to evaluate specific language domains, a number of confounding factors need to be carefully taken into account in ALS for the accurate interpretation of performance of neuropsychological batteries. These include (1) disease-specific factors such as hypercapnia, fatigue, anticholinergic medications for secretions, analgesia, poor quality sleep, dysarthria (2) concomitant neuropsychological deficits in other domains such as apathy, depression, and (3) extraneous/antecedent factors such as education, cognitive reserve (82). The complexity of linking neuropsychological manifestations to cerebral pathology is well recognised and a number of opinion papers highlighted the pitfalls to seek direct

clinico-radiological associations (83). An alternative approach to evaluate disease-burden patterns is the study of well-defined, clinically stratified patient populations with computational neuroimaging and the interrogation of cerebral regions and networks physiologically associated with language function. Accordingly, the main objective of this study is the detailed assessment of cerebral regions and networks physiologically linked to language domains and the comprehensive assessment of specific phenotypes along the ALS-FTD spectrum.

3.2 Methods

Standard Protocol Approvals, Registrations, and Patient Consents

All participants gave informed consent in accordance with the Ethics Approval of this research project (Beaumont Hospital, Dublin, Ireland - IRB).

Participants and neuroimaging

A total of 297 participants, 184 patients and 113 healthy controls (HC) were included into this study. The patient populations (**Table 3.1**) consisted of cognitively not impaired patients with ALS (“ALS_NCI”, N = 99), ALS-FTD patients not carrying the hexanucleotide repeat expansion in *C9orf72* (“ALS_FTD_C9–”, N = 29), ALS-FTD patients carrying the repeat expansion in the *C9orf72* (“ALS_FTD_C9+”, N = 24), behavioral-variant FTD patients (“bvFTD”, N = 10), patients with non-fluent variant PPA (“nfvPPA”, N = 14) and semantic variant PPA (“svPPA”, N = 7). Patients with prior neurovascular syndromes, established psychiatric diagnoses, prior neurosurgery and those unable to tolerate RMI scanning were excluded. Basic demographic and clinical data were carefully recorded for each participant on the day of MR scanning (age, sex, handedness, years of education, medications, body region of symptom onset, family history of ALS or FTD). MR data were acquired on the same 3 Tesla Philips Achieva platform. For clinical interpretation, FLAIR images were individually reviewed for each participant to identify unsuspected neurovascular or neuroinflammatory conditions. FLAIR imaging took place in axial orientation using an Inversion Recovery Turbo Spin Echo (IR-TSE) sequence: TR/TE = 11,000 / 125 ms, TI = 2800 ms, FOV = 230 × 183 × 150 mm, spatial resolution = 0.65 × 0.87 × 4 mm, 120° refocusing pulse, with flow compensation and motion smoothing and a saturation slab covering the neck region. Three different MR modalities were acquired for quantitative data interpretation; structural T1-weighted (T1w) imaging, resting-state functional MRI (rs-fMRI) and diffusion-weighted images (DWI). Some patients could not tolerate the duration of the entire protocol (**Table 3.1**), therefore not all three modalities were available for each patients. T1w data were acquired with a 3D Inversion Recovery prepared Spoiled Gradient Recalled echo (IR-SPGR) sequence with the following imaging parameters: field-of-view (FOV) of 256 × 256 × 160 mm, 160 sagittal slices with no interslice gap, flip angle (FA) = 8°, voxel resolution (VR) = 1 mm isotropic, SENSE factor = 1.5, TR/TE = 8.5/3.9 ms, TI = 1060 ms. DWI data were acquired with a spin-echo echo planar imaging (SE-EPI) pulse sequence using a 32-direction Stejskal-Tanner diffusion encoding scheme with b-values of 0 / 1100 s/mm² to record DWI data with the following settings: FOV = 245 × 245 × 150

Table 3.1 Demographic details of the study population.

	<i>Patient subgroups</i>						<i>All patients</i>	<i>HC</i>	<i>T-test [W]⁺ / Chi-square [C2]⁺⁺</i>
	<i>ALS_NCI</i>	<i>ALS_FTD_C9–</i>	<i>ALS_FTD_C9+</i>	<i>bvFTD</i>	<i>nfvPPA</i>	<i>svPPA</i>			
Number of subjects	99	29	24	10	15	7	184	113	<i>n.a.</i>
Complete T1w data	99	29	24	10	15	7	184	113	<i>n.a.</i>
Complete DWI data	94	29	22	10	13	5	173	111	<i>n.a.</i>
Complete fMRI data	96	26	22	7	8	2	161	111	<i>n.a.</i>
Age [y, mean ± SD]	58.73 ± 12.06	63.66 ± 11.13	55.50 ± 10.72	63.40 ± 6.43	71.33 ± 5.93	68.57 ± 6.29	60.74 ± 11.54	59.36 ± 10.66	W: t(251.47) = 1.04, <i>p</i> = 0.30
Sex, F / M	36 / 63	7 / 22	9 / 15	4 / 6	9 / 6	4 / 3	69 / 115	57 / 56	C2: $\chi^2(1, N = 297) = 4.29$, <i>p</i> = 0.04*
Handedness, R/L	90 / 9	26 / 3	20 / 4	10 / 0	15 / 0	7 / 0	168 / 16	106 / 7	C2: $\chi^2(1, N = 297) = 0.31$, <i>p</i> = 0.58
Years of education [mean ± SD]	13.62 ± 3.07	13.45 ± 4.21	14.04 ± 3.51	12.20 ± 4.18	13.00 ± 2.65	15.86 ± 2.20	13.60 ± 3.35	14.68 ± 3.52	W: t(228.11) = -2.61, <i>p</i> = 0.01*

Abbreviations: ALS = amyotrophic lateral sclerosis, ALS_FTD_C9–(+) = (non-)carriers of the repeat expansion in the C9ORF72 gene, ALS_NCI = non-cognitively impaired ALS, bvFTD = behavioral variant FTD, DWI = diffusion-weighted imaging, F = female, fMRI = functional MRI, FTD = frontotemporal dementia, HC = healthy control, L = left-handed, M = male, MRI = magnetic resonance imaging, N = sample size, nfv = non-fluent variant PPA, n.a. = not applicable, PPA = primary progressive aphasia, R = right-handed, SD = standard deviation, svPPA = semantic variant PPA, y = years

Symbols: ⁺ = Welch two-sample t-tests were performed to test differences of age and years of education between all patients vs. HC, ⁺⁺ = Chi-square tests were performed to test differences of sex and handedness frequencies between all patients vs. HC, * = significant at an alpha-level of *p* ≤ 0.05

mm, 60 axial slices with no interslice gaps, FA = 90°, VR = 2.5 mm isotropic, SENSE factor = 2.5, TR/TE = 7639 / 59 ms, dynamic stabilisation and spectral presaturation with inversion recovery (SPIR) fat suppression. To investigate fluctuations of the blood oxygen level dependent (BOLD) signal for functional imaging, an echo-planar imaging (EPI) sequence was utilised. 220 volumes were acquired with the following parameters: FOV = 233 × 233 × 120 mm, 30 axial slices with no interslice gap, FA = 90°, VR = 2.875 × 2.875 × 4 mm isotropic, SENSE factor = 2.5, TR/TE = 2000 / 35 ms, acquisition time: 5 min 41 s, pixel bandwidth ~ 1900, Hz/Px (with slight variations of up to 150 Hz/Px between subjects).

Cortical thickness estimation of Broca’s and Wernicke’s Areas

Cortical thickness (CT) was estimated in Broca’s and Wernicke’s areas based on structural T1w data. T1w data were first pre-processed using FreeSurfer’s (8) automated “recon_all” pipeline, which – in brief – includes bias correction, brain extraction, normalization and renders the data onto a 2D surface representation of the cortex, among others (84, 85). To facilitate data management for further downstream analyses, surface data into were converted into “CIFTI” file format using Ciftify (86) which uses tools from Workbench (87). Additionally, the data were also pre-processed using the standard “fsl_anat” pipeline of FMRIB’s Software Library (FSL) (12). This, in brief, includes bias correction, brain extraction and normalization to the MNI152 2mm standard space by means of non-linear registration, and outputs transformation matrices to be subsequently used for downstream image co-registration of DWI and functional data. “Broca’s area” was defined as the merged “pars opercularis” and “pars triangularis” labels of the Desikan-Killiany (DK) atlas (88). Wernicke’s Area was defined based on the “banks of the superior temporal sulcus” of the DK atlas – encompassing parts of the superior temporal gyrus and posterior middle temporal gyrus, which is an topological approximation of Wernicke’s Area (89) (**Figure 3.1**). Language is lateralised to the left hemisphere in right handed subjects and also thought to be lateralised to the left in most left handed people (90, 91). However, we extracted Broca’s and Wernicke’s areas, i.e. their contralateral right hemispheric analogues, to further evaluate inter-hemispheric differences. CT was defined as a single value for each ROI and subject, i.e. the average of all vertices comprising that respective ROI.

Structural connectivity estimation of six language-associated tracts

Structural connectivity (SC) was estimated based on diffusion-weighted (DW) data. First, data were pre-processed using tools from MRtrix3 (16), which included noise removal (92), removal of

Gibb’s Ringing artifacts (18), motion and eddy current corrections (19) as well as bias field correction (20).

As integrity metrics of white tracts classically associated with language function were investigated, the following six fibre tracts were specifically assessed in each hemisphere; the arcuate fascicle (AF), inferior occipito-frontal fascicle (IFO), inferior longitudinal fascicle (ILF), superior longitudinal fascicle (SLF), uncinate fascicle (UF) (93, 94) as well as the frontal aslant tract (FAT), which connects the supplementary motor region / lateral superior frontal gyrus to the inferior frontal gyrus (95). The segmentation pipeline TractSeg (96) was used to identify AF, IFO, ILF, SLF, and UF which uses a neural network algorithm to segment individual DWI data sets. TractSeg outputs three separate fibre bundles for SLF, which we merged into a single SLF map. The advantage of the neural-network-based method is that it does not assume anatomical commonality between subjects, and detects individual WM microstructure variations. The constrained spherical deconvolution (CSD) method was implemented to estimate fibre orientation distribution (fODF) at each voxel and peaks of the spherical harmonic function were extracted using tools from MRtrix3 (16). CSD is increasingly utilised instead of the tensor model to estimate fibre orientation and perform tractography since it may outperform the tensor model in crossing fibres regions (97-99). CSD requires the estimation of a so-called “response function”, which we provided using the dhollander method as implemented in MRtrix3. Even though DW data were only acquired with a b-value of 1100 s/mm², the additionally recorded b = 0 s/mm² images allowed to implement a multishell approach nevertheless. Resulting fODFs were normalized according to Raffelt et al. (100); spherical harmonic peaks were retrieved from the normalised measures, which then served as input values into TractSeg. This segmentation strategy could not be implemented in some subjects and tracts, therefore the total number of subjects varied slightly between the tracts, as well as the relevant degrees of freedom (DOFs) as presented in **Table 3.2**. For the segmentation of the frontal aslant tract, we first defined the source and target ROIs based on the available literature (95) using the labels of the Glasser atlas (101) in volumetric space. We aligned labels and DW images to the high-resolution T1w data and calculated tractograms between each pair of ROIs using a probabilistic algorithm, generating 5000 streamlines per tract, using the analogue options and parameters for estimating fODF and ultimately tractography as for TractSeg. The 12 track images, six in each hemisphere, were then mapped onto track-weighted images using the track density imaging (TDI) method (102), where each streamline contributes a value of unity to the final track-weighted output map. We binarised this map using a threshold of at minimum two streamlines per voxel. With the resulting binarized maps, structural connectivity could be

estimated for each tract, which was defined as mean values of radial diffusivity (RD) and fractional anisotropy (FA) of all voxels comprising the respective tract. Both RD and FA are considered to reflect aspects of WM microstructure and are derivatives of the tensor model, which we calculated by means of least squares estimation (103).

Functional connectivity between Broca and Wernicke areas and along the frontal aslant tract

To evaluate any potential discriminative utility of functional connectivity (FC) to differentiate patient groups, we estimated FC for two ROI pairs, namely FC between Broca’s and Wernicke’s areas as well as FC along FAT (i.e. between the supplementary motor region / lateral superior frontal gyrus and the inferior frontal gyrus). FC was calculated for each subject in MNI152 2mm standard space using FSL’s feat pipeline, including brain extraction, slice-time correction, and motion correction. Correction of head-motion-related artifacts was added in a separate data preparation step using FSL’s AROMA algorithm (14). Each patient’s pre-processed, de-noised functional image was standardized to the MNI152 2mm standard space for high-level group comparisons in a two-step procedure: (1) linear co-registration of native high-resolution data using 6 DOFs, followed by (2) non-linearly warping into standard space using 12 DOFs. FC was then defined as Fisher z-transformed Pearson’s correlation between the mean time courses of the above specified ROIs, separately in the two hemispheres. FC was calculated FC in Matlab R2021b (The Mathworks, Natick, USA), making use of CoSMoMVPA (15) and FieldTrip (104) toolboxes.

Statistical modelling

Statistical analyses were conducted with RStudio (R version 4.2.2). Differences in means of age and education between patients (aggregating across subgroups) and HC were examined using Welch two-sample t-tests, whereas sex / handedness frequencies were compared using Chi-square testing. To test differences in neuroimaging metrics between the patient cohorts and HC, a one-way analysis of variance (ANOVA) omnibus test was implemented, correcting for the confounding effects of age, sex, handedness and years of education. When the omnibus test was significant at an alpha level of $p \leq 0.05$, post-hoc pairwise differences of means were explored using Tukey’s Honestly Significance Test (HSD) generating p-values corrected for the family-wise error rate (FWER). Our initial results suggested that RD may better discriminate between the subgroups compared to FA in terms of SC. To further test this, another chi-square test was implemented comparing the frequencies of significant pairwise contrasts in Tukey’s HSD tests (**Table 3.2**). Specifically, we compared the counts of all possible significant post-hoc contrasts (21

subject group combinations x 6 tracts x 2 hemispheres = 252 possible contrasts) and observed significant post-hoc contrasts for RD and FA respectively.

3.3 Results

Demographics

Descriptive demographic data are summarised in **Table 3.1** While age ($t(251.47) = 1.04$, $p = 0.30$) and handedness ($\chi^2(1, N = 297) = 0.31$, $p = 0.58$) was adequately matched, the aggregated patient group differed from the HC group in terms of gender distribution ($\chi^2(1, N = 297) = 4.29$, $p = 0.04$) – suggesting a higher relative amount of females across the patient subgroups – and years of education ($t(228.11) = -2.61$, $p = 0.01$), indicative of longer education in HC. For downstream statistical modelling, all four variables were accounted for to correct for their potential confounding effects.

Cortical thickness

Cortical thickness (CT) of Broca’s area differed significantly between the groups ($F(6,286) = 23.90$, $p < .001^*$), and post-hoc testing revealed lower values of all six patient subgroups compared to controls (**Figure 3.1A, Table 3.2**) ALS_NCI group exhibited higher values compared to most patient subgroups other than svPPA. Analogous results were found for this cortical region in the right hemisphere. Wernicke’s area thickness (**Figure 3.1B**) also showed group differences ($F(6,286) = 13.22$, $p < .001^*$) and HC displayed higher values vs. most patient subgroups except ALS_NCI and bvFTD. NfvPPA patients exhibited lower values compared to other groups except ALS_FTD_C9+ and svPPA. For the equivalent region in the right hemisphere, lower values were identified in most patient groups (except svPPA) compared to HC.

Structural connectivity

Structural connectivity (SC) of each language-associated tracts showed group differences. RD captured more significant pairwise contrasts in post-hoc testing. Statistical details are presented in

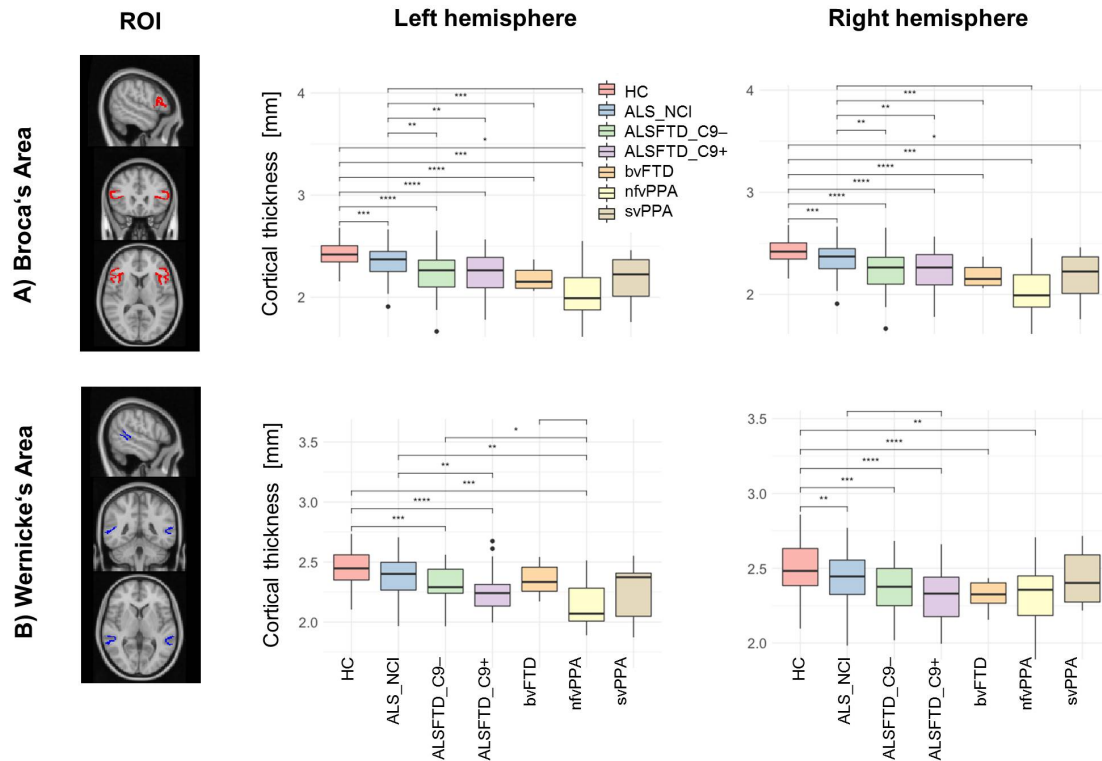


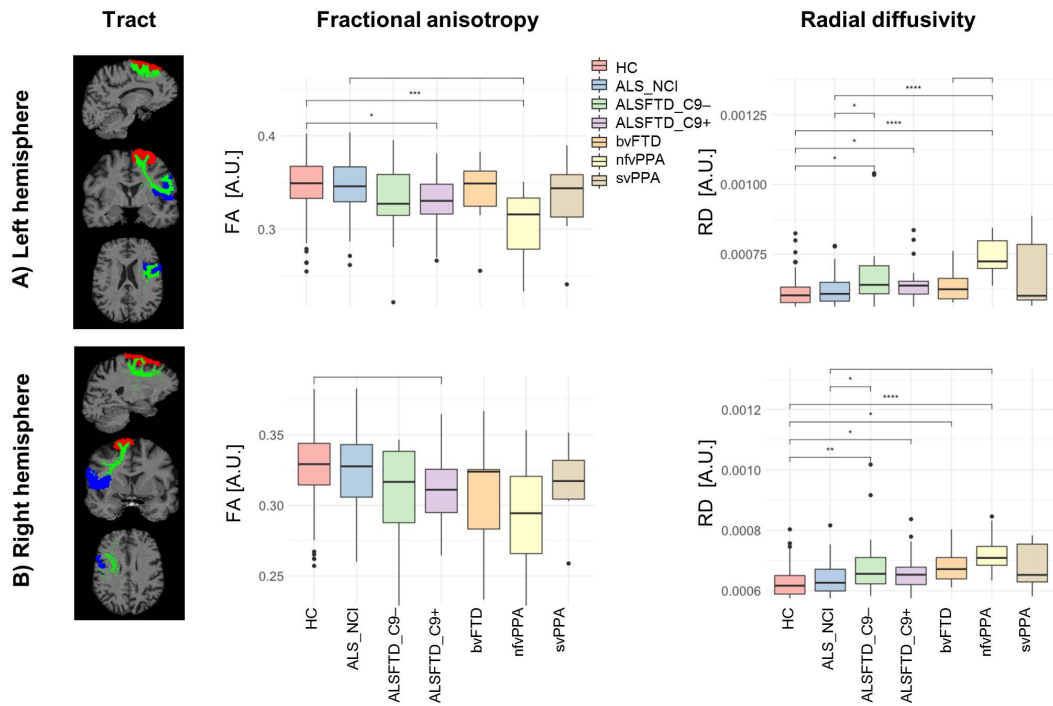
Table 3.2 and the most significant findings are listed below. The FAT (**Figure 3.2**) was affected in most patient cohorts in both hemispheres compared to controls. The ALS_NCI group was the least affected patient group. NfvPPA patients were the most affected, followed by ALS_FTD_C9+. The AF was most affected in nvPPA, and to a lesser degree in ALS_FTD_C9+ patients. This was especially evident for RD and seemed specific for the left hemisphere (**Figure 3.3A**). Left-hemispheric IFO was not affected in bvFTD, nvPPA and ALS_NCI compared to controls but

Figure 3.1 Differences of cortical thickness (CT) of Broca's Area (A, ROI shown in red on MNI152 2mm standard space) and Wernicke's Area (B, ROI shown in blue) were evaluated between the patient subgroups and controls using ANOVA omnibus tests. For both ROIs and their right-hemispheric equivalents, a significant omnibus test suggested a main effect of group which we further explored in Tukey's HSD testing. Significant results of those post-hoc comparisons are indicated as brackets between the respective groups (asteriks represent different levels of significance: " + " – $p_{adj} \leq 0.05$; " * " – $p_{adj} \leq 0.01$; " ** " – $p_{adj} \leq 0.001$). Box plots indicate medians and upper / lower quartiles, as well as outliers as dots. Abbreviations: adj = adjusted, ALS = amyotrophic lateral sclerosis, ALS_FTD_C9–(+) = (non-)carriers of the repeat expansion in the C9ORF72 gene, ALS_NCI = non-cognitively impaired ALS, ANOVA = analysis of variance, bvFTD = behavioral variant FTD, CT = cortical thickness, FTD = frontotemporal dementia, HC = healthy control, HSD = honest significant difference, MNI = Montreal Neurological Institute, nvf = non-fluent variant PPA, PPA = primary progressive aphasia, ROI = region of interest, svPPA = semantic variant PPA

affected in ALS_FTD_C9+/- and svPPA as measured by RD. The svPPA group tended to be the most affected patient group. Left-hemispheric ILF of svPPA patients was the worst affected, since it showed significantly higher mean RD values vs. all other groups in post-hoc testing (**Figure 3.3C**). In the right hemisphere, the relative higher disease burden of svPPA was evident but not as pronounced as on the left hemisphere. While RD did not vary for the SLF on neither hemisphere (**Figure 3.3D**), FA suggested that the nvPPA group was the worst affected patient subgroup for the left hemisphere, yielding significantly lower values as compared to HC, ALS_NCI and

Figure 3.2 . Group differences of structural connectivity (SC) of the left (A) and right (B) frontal aslant tracts were suggested by significant ANOVA omnibus tests for both fractional anisotropy (middle column) and radial diffusivity (right column). We illustrate those tracts for an exemplary subject in the left column in green and provide the “source” and “target” ROIs in red and blue. The main effects were further explored in Tukey’s HSD testing. Significant results of those post-hoc comparisons are indicated as brackets between the respective groups (asteriks represent different levels of significance: “ + ” – $p_{adj} \leq 0.05$; “ * ” – $p_{adj} \leq 0.01$; “ ** ” – $p_{adj} \leq 0.001$). Box plots indicate medians and upper / lower quartiles, as well as outliers as dots. Abbreviations: adj = adjusted, ALS = amyotrophic lateral sclerosis, ALS_FTD_C9-(+) = (non-)carriers of the repeat expansion in the C9ORF72 gene, ALS_NCI = non-cognitively impaired ALS, ANOVA = analysis of variance, bvFTD = behavioral variant FTD, FA = fractional anisotropy, FTD = frontotemporal dementia, HC = healthy control, HSD = honest significant difference, nvf = non-fluent variant PPA, PPA = primary progressive aphasia, RD = radial diffusivity, ROI = region of interest, SC = structural connectivity, svPPA = semantic variant PPA

ALS_FTD_C9-. In the right hemisphere, however, the ALS_FTD_C9+ group was the most affected group, yielding lower values as compared to all other groups but svPPA. Finally, the UF was mostly affected in the svPPA group with higher RD as compared to most other subgroups and for both



hemispheres (**Figure 3.3E**). Moreover, we observed that HC and ALS_NCI yielded comparable results and overall similar trends in post-hoc testing. Across all tracts in both

hemispheres, RD analyses yielded to more significant post-hoc pairwise contrasts (N=77) than FA (N=13) (**Table 3.2**) ($X^2(1, N = 504) = 53.69, p < 0.001$).

Functional connectivity

Functional connectivity (FC) between Broca’s and Wernicke’s areas does not differentiate patient groups either in the left ($F(6,261) = 1.71, p=0.12$) or right hemisphere ($F(6,261) = 1.22, p=0.30$) (**Figure 3.4A**). FC along FAT does exhibit group differences ($F(6,261) = 3.41, p=.003$), but the only significant pairwise contract post-hoc is ALS_NCI exhibiting lower FC compared to controls ($p_{adj} = 0.001$). No similar pattern was identified in the right hemisphere ($F(6,261) = 1.54, p=0.17$) (**Figure 3.4B**).

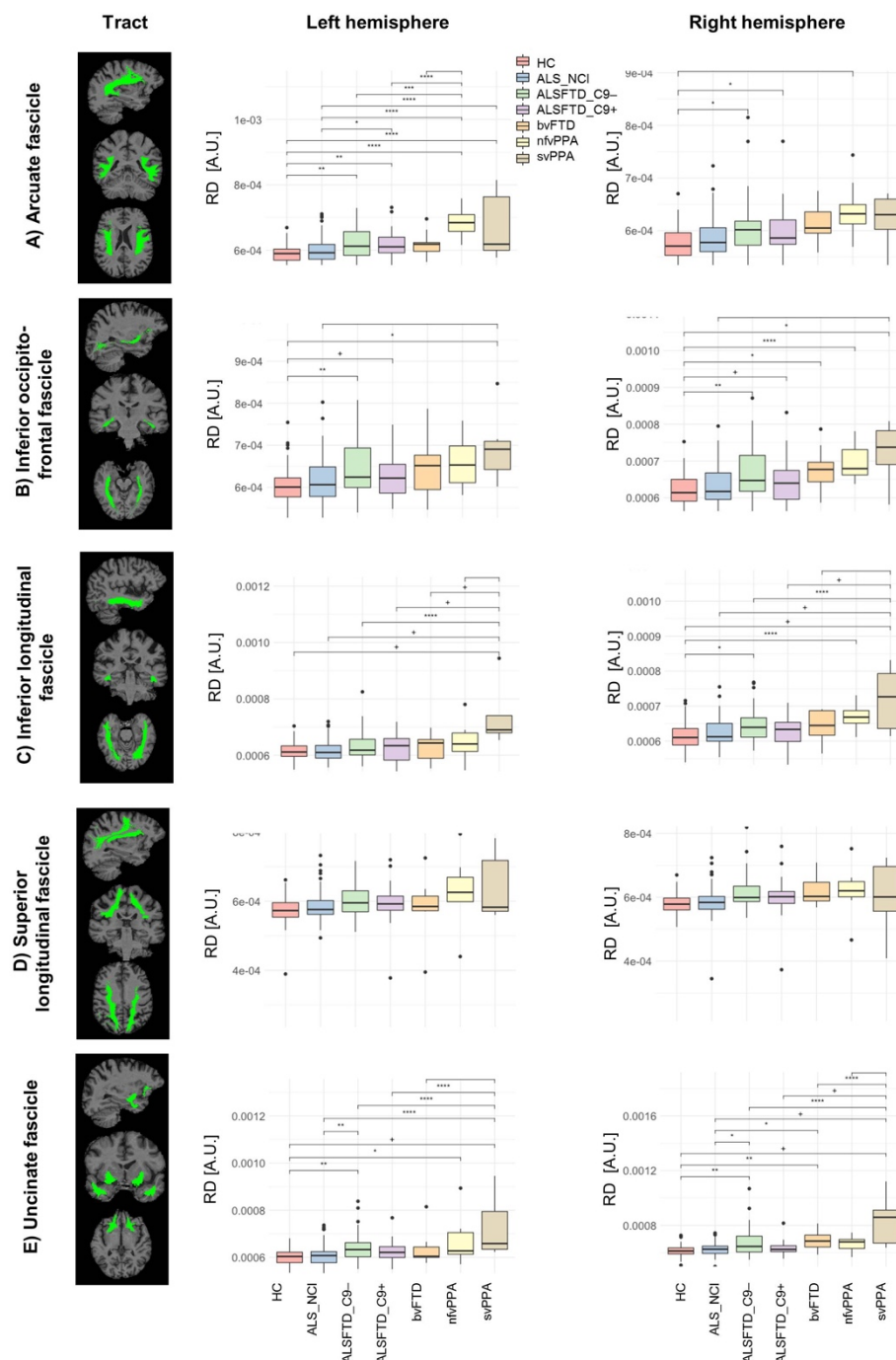


Figure 3.3 Structural connectivity (SC) of the remaining five tracts (depicted in green for an exemplary subject in the left column) was mostly found as variant between the groups by a significant ANOVA omnibus test. We evaluated SC as both radial diffusivity (RD) and fractional anisotropy (FA), but show here only RD since it yielded more significant pairwise contrast in post-hoc Tukey’s HSD testing as compared to FA (cf. also **Table 3.1**). We indicate significant post-hoc comparisons as brackets between the respective groups (asteriks represent different levels of significance: “+” – $\text{padj} \leq 0.05$; “*” – $\text{padj} \leq 0.01$; “**” – $\text{padj} \leq 0.001$). Box plots indicate medians and upper / lower quartiles, as well as outliers as dots. Abbreviations: adj = adjusted, AF = arcuate fascicle ALS = amyotrophic lateral sclerosis, ALS_FTD_C9–(+) = (non-)carriers of the repeat expansion in the C9ORF72 gene, ALS_NCI = non-cognitively impaired ALS, ANOVA = analysis of variance, bvFTD = behavioral variant FTD, FA = fractional anisotropy, FTD = frontotemporal dementia, IFO = inferior occipito-frontal fascicle, ILF = inferior longitudinal fascicle, HC = healthy control, HSD = honest significant difference, nvf = non-fluent variant PPA, PPA = primary progressive aphasia, RD = radial diffusivity, ROI = region of interest, SC = structural connectivity, SLF = superior longitudinal fascicle, svPPA = semantic variant PPA, UF = uncinate fascicle

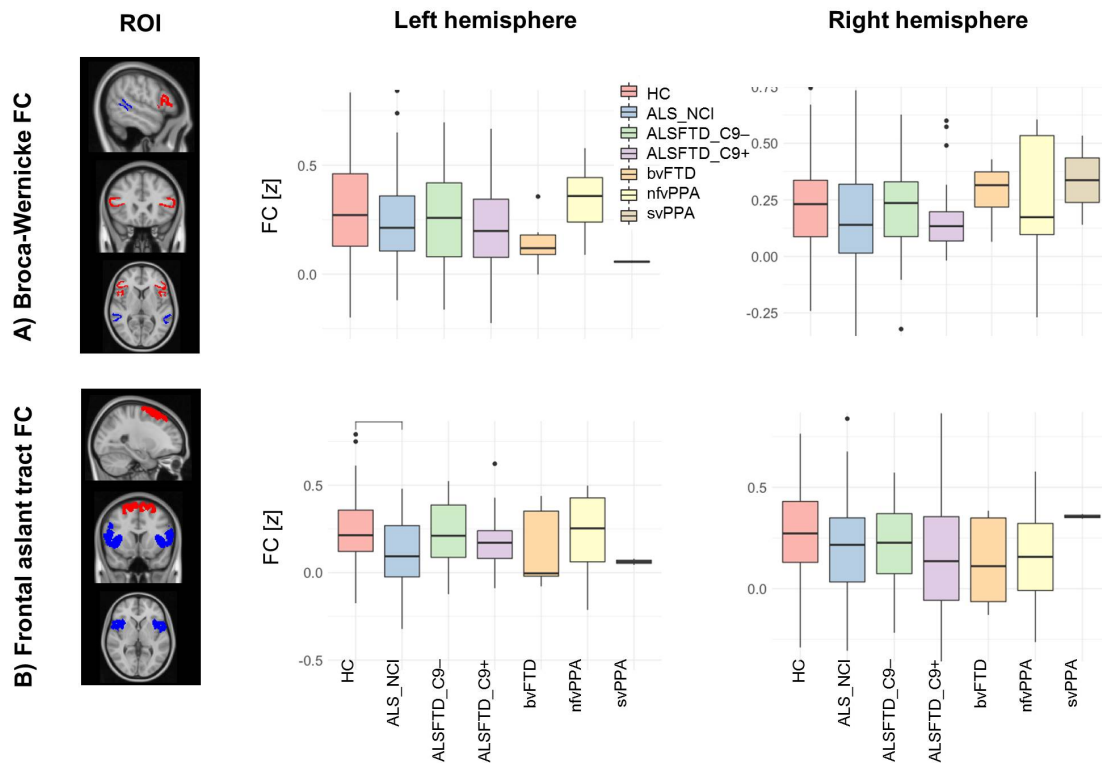


Figure 3.4 Functional connectivity (FC) between Broca's (A, red) and Wernicke's Areas (A, blue), i.e. their right-hemispheric equivalents, did not yield significant group differences in the ANOVA omnibus test. Similarly, FC of the frontal aslant tract, i.e. FC between its source (B, red) and target (B, blue) ROIs did not vary between the groups for the right but the left hemisphere, whereas post-hoc Tukey HSD testing revealed significantly lower FC for ALS_NCI as compared to HC (indicated as bracket). ROIs are shown on MNI152 2mm standard space in the left column. Box plots indicate medians and upper / lower quartiles, as well as outliers as dots. Abbreviations: ALS = amyotrophic lateral sclerosis, ALS_FTD_C9–(+) = (non-)carriers of the repeat expansion in the C9ORF72 gene, ALS_NCI = non-cognitively impaired ALS, ANOVA = analysis of variance, bvFTD = behavioral variant FTD, FA = fractional anisotropy, FTD = frontotemporal dementia, HC = healthy control, HSD = honest significant difference, MNI = Montreal Neurological Institute, nvf = non-fluent variant PPA, PPA = primary progressive aphasia, RD = radial diffusivity, ROI = region of interest, svPPA = semantic variant PPA

Table 3.2 Statistical comparisons of neuroimaging metrics between FTD patients vs. healthy controls and post-hoc comparisons among subject subgroups.

	One-way ANOVA (omnibus test, main effect: “diagnosis”)			
	Left hemisphere		Right hemisphere	
	F-value (DOF), <i>p</i> -value	<i>p</i> -value Significant pairwise contrasts (post-hoc) [<i>p</i> -value]	F-value (DOF), <i>p</i> -value	Significant pairwise contrasts (post-hoc) [<i>p</i> -value]
Cortical thickness				
Broca’s Area	$F(6,286)=23.90$, $p<.001^*$	- HC – ALS_NCI [$p_{adj} = 0.003$] - HC – ALSFTD_C9– [$p_{adj} < 0.001$] - HC – ALSFTD_C9+ [$p_{adj} < 0.001$] - HC – bvFTD [$p_{adj} < 0.001$] - HC – nfVPPA [$p_{adj} < 0.001$] - HC – svPPA [$p_{adj} = 0.012$] - ALS_NCI – ALSFTD_C9– [$p_{adj} = 0.023$] - ALS_NCI – ALSFTD_C9+ [$p_{adj} < 0.001$] - ALS_NCI – bvFTD [$p_{adj} = 0.032$] - ALS_NCI – nfVPPA [$p_{adj} < 0.001$]	$F(6,286)=23.87$, $p<.001^*$	- HC – ALS_NCI [$p_{adj} = 0.002$] - HC – ALSFTD_C9– [$p_{adj} < 0.001$] - HC – ALSFTD_C9+ [$p_{adj} < 0.001$] - HC – bvFTD [$p_{adj} < 0.001$] - HC – nfVPPA [$p_{adj} < 0.001$] - HC – svPPA [$p_{adj} = 0.03$] - ALS_NCI – ALSFTD_C9– [$p_{adj} = 0.014$] - ALS_NCI – ALSFTD_C9+ [$p_{adj} < 0.001$] - ALS_NCI – bvFTD [$p_{adj} = 0.010$] - ALS_NCI – nfVPPA [$p_{adj} < 0.001$]
Wernicke’s Area	$F(6,286)=13.22$, $p<.001^*$	- HC – ALSFTD_C9– [$p_{adj} = 0.005$] - HC – ALSFTD_C9+ [$p_{adj} < 0.001$] - HC – nfVPPA [$p_{adj} < 0.001$] - ALS_NCI – ALSFTD_C9+ [$p_{adj} = 0.006$] - ALSFTD_C9– – nfVPPA [$p_{adj} = 0.035$] - bvFTD – nfVPPA [$p_{adj} = 0.033$]	$F(6,286)=9.10$, $p<.001^*$	- HC – ALS_NCI [$p_{adj} = 0.023$] - HC – ALSFTD_C9– [$p_{adj} = 0.010$] - HC – ALSFTD_C9+ [$p_{adj} < 0.001$] - HC – bvFTD [$p_{adj} = 0.020$] - HC – nfVPPA [$p_{adj} = 0.004$] - ALS_NCI – ALSFTD_C9+ [$p_{adj} = 0.007$]
Structural connectivity: Frontal aslant tract (FAT)				
FA	$F(6,271)=6.05$, $p<.001^*$	- HC – ALSFTD_C9+ [$p_{adj} = 0.030$] - HC – nfVPPA [$p_{adj} = 0.012$] - ALS_NCI – nfVPPA [$p_{adj} = 0.036$]	$F(6,271)=5.78$, $p<.001^*$	- HC – ALSFTD_C9+ [$p_{adj} = 0.023$]
RD	$F(6,271)=12.99$, $p<.001^*$	- HC – ALSFTD_C9– [$p_{adj} < 0.001$] - HC – ALSFTD_C9+ [$p_{adj} = 0.023$] - HC – nfVPPA [$p_{adj} < 0.001$] - ALS_NCI – ALSFTD_C9– [$p_{adj} = 0.017$] - ALS_NCI – nfVPPA [$p_{adj} = 0.017$] - nfVPPA – bvFTD [$p_{adj} < 0.001$]	$F(6,271)=11.95$, $p<.001^*$	- HC – ALSFTD_C9– [$p_{adj} < 0.001$] - HC – ALSFTD_C9+ [$p_{adj} = 0.011$] - HC – bvFTD [$p_{adj} = 0.0281$] - HC – nfVPPA [$p_{adj} < 0.001$] - ALS_NCI – ALSFTD_C9– [$p_{adj} = 0.016$] - ALS_NCI – nfVPPA [$p_{adj} = 0.002$]
Structural connectivity: Arcuate fascicle (AF)				
FA	$F(6,282)=5.50$, $p<.001^*$	- HC – ALSFTD_C9+ [$p_{adj} = 0.006$]	$F(6,282)=2.38$, $p=.03^*$	– –
RD	$F(6,271)=21.85$, $p<.001^*$	- HC – ALSFTD_C9– [$p_{adj} = 0.003$] - HC – ALSFTD_C9+ [$p_{adj} < 0.001$] - HC – nfVPPA [$p_{adj} < 0.001$] - HC – svPPA [$p_{adj} = 0.012$] - ALS_NCI – ALSFTD_C9+ [$p_{adj} = 0.032$]	$F(6,282)=9.89$, $p<.001^*$	- HC – ALSFTD_C9– [$p_{adj} < 0.001$] - HC – ALSFTD_C9+ [$p_{adj} < 0.001$] - HC – nfVPPA [$p_{adj} < 0.001$]

Chapter 2: “Language-associated brain regions in ALS-FTD”

		- ALS_NCI – nfvPPA [$p_{\text{adj}} < 0.001$] - ALS_NCI – svPPA [$p_{\text{adj}} = 0.001$] - ALSFTD_C9– – nfvPPA [$p_{\text{adj}} < 0.001$] - ALSFTD_C9+ – nfvPPA [$p_{\text{adj}} = 0.013$] - bvFTD – nfvPPA [$p_{\text{adj}} = 0.008$]		
Structural connectivity: Inferior occipito-frontal fascicle (IFO)				
FA	$F(6,281)=$ 0.76, $p=0.60$	<i>n.a.</i>	$F(6,280)=$ 1.13, $p=0.35$	<i>n.a.</i>
RD	$F(6,281)=$ 9.35, $p<.001^*$	- HC – ALSFTD_C9– [$p_{\text{adj}} = 0.028$] - HC – ALSFTD_C9+ [$p_{\text{adj}} = 0.018$] - HC – svPPA [$p_{\text{adj}} = 0.003$] - ALS_NCI – svPPA [$p_{\text{adj}} = 0.026$]	$F(6,280)=$ 14.48, $p<.001^*$	- HC – ALSFTD_C9– [$p_{\text{adj}} = 0.001$] - HC – ALSFTD_C9+ [$p_{\text{adj}} = 0.005$] - HC – bvFTD [$p_{\text{adj}} = 0.030$] - HC – nfvPPA [$p_{\text{adj}} = 0.010$] - HC – svPPA [$p_{\text{adj}} = 0.003$] - ALS_NCI – svPPA [$p_{\text{adj}} = 0.005$]
Structural connectivity: Inferior longitudinal fascicle (ILF)				
FA	$F(6,278)=$ 0.70, $p=0.65$	<i>n.a.</i>	$F(6,272)=$ 0.85, $p=0.53$	<i>n.a.</i>
RD	$F(6,278)=$ 9.34, $p<.001^*$	- HC – svPPA [$p_{\text{adj}} < 0.001$] - ALS_NCI – svPPA [$p_{\text{adj}} < 0.001$] - ALSFTD_C9– – svPPA [$p_{\text{adj}} < 0.001$] - ALSFTD_C9+ – svPPA [$p_{\text{adj}} < 0.001$] - bvFTD – svPPA [$p_{\text{adj}} < 0.001$] - nfvPPA – svPPA [$p_{\text{adj}} < 0.001$]	$F(6,272)=$ 10.76, $p<.001^*$	- HC – ALSFTD_C9– [$p_{\text{adj}} = 0.045$] - HC – nfvPPA [$p_{\text{adj}} = 0.046$] - HC – svPPA [$p_{\text{adj}} < 0.001$] - ALS_NCI – svPPA [$p_{\text{adj}} < 0.001$] - ALSFTD_C9– – svPPA [$p_{\text{adj}} = 0.007$] - ALSFTD_C9+ – svPPA [$p_{\text{adj}} = 0.012$] - bvFTD – svPPA [$p_{\text{adj}} = 0.031$]
Structural connectivity: Superior longitudinal fascicle (SLF)				
FA	$F(6,282)=$ 8.40, $p=0.04^*$	- HC – nfvPPA [$p_{\text{adj}} < 0.001$] - ALS_NCI – nfvPPA [$p_{\text{adj}} < 0.001$] - ALSFTD_C9– – nfvPPA [$p_{\text{adj}} = 0.002$]	$F(6,281)=$ 8.77, $p<.001^*$	- HC – ALSFTD_C9+ [$p_{\text{adj}} < 0.001$] - ALS_NCI – ALSFTD_C9+ [$p_{\text{adj}} < 0.001$] - ALSFTD_C9– – ALSFTD_C9+ [$p_{\text{adj}} < 0.001$] - ALSFTD_C9+ – bvFTD [$p_{\text{adj}} = 0.050$] - ALSFTD_C9+ – nfvPPA [$p_{\text{adj}} = 0.023$]
RD	$F(6,282)=$ 2.24, $p<.001^*$	--	$F(6,281)=$ 3.57, $p=.002^*$	--
Structural connectivity: Uncinate fascicle (UF)				
FA	$F(6,270)=$ 1.08, $p=0.37$	<i>n.a.</i>	$F(6,273)=$ 3.16, $p=.005^*$	--
RD	$F(6,270)=$ 12.02, $p<.001^*$	- HC – ALSFTD_C9– [$p_{\text{adj}} < 0.001$] - HC – nfvPPA [$p_{\text{adj}} = 0.029$] - HC – svPPA [$p_{\text{adj}} < 0.001$] - ALS_NCI – ALSFTD_C9– [$p_{\text{adj}} = 0.010$] - ALS_NCI – svPPA [$p_{\text{adj}} < 0.001$]	$F(6,273)=$ 21.43, $p<.001^*$	- HC – ALSFTD_C9– [$p_{\text{adj}} < 0.001$] - HC – bvFTD [$p_{\text{adj}} < 0.001$] - HC – svPPA [$p_{\text{adj}} < 0.001$] - ALS_NCI – ALSFTD_C9– [$p_{\text{adj}} < 0.001$] - ALS_NCI – bvFTD [$p_{\text{adj}} = 0.006$]

		- ALSFTD_C9– – svPPA [$p_{adj} = 0.041$] - ALSFTD_C9+ – svPPA [$p_{adj} = 0.003$] - bvFTD – svPPA [$p_{adj} = 0.029$]		- ALS_NCI – svPPA [$p_{adj} < 0.001$] - ALSFTD_C9– – svPPA [$p_{adj} < 0.001$] - ALSFTD_C9+ – svPPA [$p_{adj} < 0.001$] - bvFTD – svPPA [$p_{adj} < 0.001$] - nfvPPA – svPPA [$p_{adj} < 0.001$]
Functional connectivity				
Broca-Wernicke FC	$F(6,261)=$ 1.71, $p=0.12$	<i>n.a.</i>	$F(6,261)=$ 1.22, $p=0.30$	<i>n.a.</i>
Aslant tract FC	$F(6,261)=$ 3.41, $p=.003^*$	- HC – ALS_NCI [$p_{adj} = 0.001$]	$F(6,261)=$ 1.54, $p=0.17$	<i>n.a.</i>

Statistics are provided for the main effect “diagnosis” between the patient / HC groups in a one-way ANOVA omnibus test, which was corrected for confounding effects of age, sex, years of education and handedness. Notice that the degrees of freedom vary between the comparisons for two reasons: 1) different amounts of complete data for the different MRI sequences (T1w / DWI / rs-fMRI, cf. also Table 3.1); 2) segmentation errors for specific tracts of DWI data (we used an automated, AI-based algorithm to segment data in individual space which occasionally failed for some tracts in some individuals, cf. methods section). When the overall ANOVA omnibus test was significant, we proceeded with post-hoc testing to identify significant pairwise contrasts using Tukey’s HSD tests, which we provide in this table (we report only significant contrasts; notice that the resulting p -values in Tukey’s HSD tests are corrected for family-wise error rate, which we indicate as “ p_{adj} ”).

Abbreviations/Symbols: adj = adjusted, AI = artificial intelligence, ALS = amyotrophic lateral sclerosis, ALS_FTD_C9–(+) = (non-)carriers of the repeat expansion in the C9ORF72 gene, ALS_NCI = non-cognitively impaired ALS, ANOVA = analysis of variance, bvFTD = behavioral variant FTD, DOF = degrees of freedom, DWI = diffusion-weighted imaging, FA = fractional anisotropy, FC = functional connectivity, FTD = frontotemporal dementia, HC = healthy control, HSD = honest significant difference, nfv = non-fluent variant PPA, *n.a.* = not applicable (*here: ANOVA omnibus test was not significant and therefore post-hoc testing was not justified*), PPA = primary progressive aphasia, RD = radial diffusivity, rs-fMRI = resting-state functional magnetic resonance imaging, svPPA = semantic variant PPA, T1w = T1-weighted, “*” = significant at an alpha-level of $p \leq 0.05$, “– –” = post-hoc testing yielded no significant pairwise comparisons in spite of overall significant ANOVA omnibus test.

3.4 Discussion

Our results not only confirm the significant pathology of language-associated brain regions in language variant FTD phenotypes (105), but also showcase the pathology of these regions in ALS. Neuroimaging signatures in FTD are very well characterised in the literature (106), but it is noteworthy that in our analyses, Wernicke’s area is most affected in nvPPA and unaffected in svPPA compared to controls. Patients with nvPPA exhibited the lowest left hemispheric cortical thickness in both Broca’s and Wernicke’s area. Similarly the integrity of the frontal aslant tract was the most affected in this group. Interestingly, our cohort of bvFTD exhibits bi-hemispheric Broca’s area pathology and cortical involvement in the right-hemispheric equivalent of Wernicke’s. From a tractography point-of-view, the bvFTD group also exhibits RD changes in the right frontal aslant tract, right inferior occipito-frontal fascicle and right uncinate compared to healthy controls.

The main finding of this study is the demonstration that ALS cohorts also show varying degree of language-region involvement depending on their genotype and phenotype. The gene-negative ALSnci group shows bi-hemispheric Broca’s area atrophy, as well as cortical thinning in the right-hemispheric equivalent of Wernicke’s area. Despite the detected cortical atrophy in ALSnci the white matter tracts evaluated showed no degenerative change in this cohort compared to controls. However, ALSnci patients exhibit lower functional connectivity along the FAT than controls. Both ALS-FTD cohorts exhibit Broca’s and Wernicke’s area atrophy in the left hemisphere as well as in their contralateral equivalents. The *C9orf72* positive ALS-FTD cohort is the only ALS group with bilateral FA reduction along the frontal aslant tract, but FAT RD is increased bilaterally in both ALS-FTD cohorts irrespective of *C9orf72* status. Both ALS-FTD groups show arcuate and inferior occipito-frontal pathology bilaterally based on RD. *C9orf72* positive ALS-FTD patients show additional right superior longitudinal fascicle (SLF) involvement. Interestingly only the *C9* negative ALS-FTD group demonstrates bilateral uncinate and right inferior longitudinal fascicle involvement, while *C9* positive ALS-FTD patients don’t (**Table 3.2, Figure 3.4**).

Our analyses highlight that focal structural changes alone do not account for clinical symptoms and instead of solely performing voxelwise analyses, the integrity of specific networks and tracts should be also evaluated (107, 108). The shift from focality to circuitry has been repeatedly emphasised in ALS (109-111) and is consistent with the observation that specific neuropsychological functions are mediated by multi-synaptic networks with distinct cortical and white matter components many relayed through specific subcortical nuclei and sometimes

modulated by cerebellar afferents (112, 113). The concomitant degeneration of inter-connected grey matter regions and their association fibres is demonstrated by the ALS-FTD groups where the cortical degeneration of Broca’s and Wernicke’s areas are accompanied by the interconnecting arcuate fibres. While only supratentorial language regions were evaluated in this study, it is increasingly recognised that the cerebellum also play a physiological role in language. Cerebellar pathology is well characterised in both ALS and PLS and has been confirmed by a series of radiology and post mortem studies (7, 114, 115) and also recognised in FTD (116). From a methodological perspective, the value of running complementary structural functional analyses is well illustrated by this study. Functional MRI has been extensively applied I ALS cohorts generating valuable academic insights (39). Early fMRI studies in ALS were paradigm based studies focusing on motor regions, which were increasingly superseded by resting-state and more recently a series of elegant motor imagery studies have also been published. The benefit of rsfMRI lies in its ability to appraise connectivity along complex multi-synaptic networks, but the potential confounds of underlying vascular pathology, vasoactive and psychoactive medications have to be taken into account. This notion is supported by our findings of left hemispheric functional connectivity reduction between the end-points of the frontal aslant track in cognitively not-impaired ALS patients, where traditional tractography did not detect white matter alterations. This demonstrates the benefit of evaluating a panel of structural and functional connectivity indices in motor neuron diseases as they may have divergent sensitivity to detect network degeneration (30, 70, 107, 117-119). Structural connectivity is typically appraised based on diffusion data sets, which have their own caveats particularly around regions of crossing fibres. The most commonly evaluated diffusivity measure is FA, but as demonstrated in our study it may have a limited detection sensitivity compared to other measures such as RD. In our dataset, with the exception of the superior longitudinal fascicle, RD identified more significant group differences than FA. Novel diffusion models and high angular resolution diffusion-weighted imaging are increasingly implemented in ALS and FTD imaging as they are more likely to overcome the challenges associated with crossing fibres (35, 120, 121).

Even though extrapyramidal, cerebellar and frontotemporal deficits and their corresponding radiological changes are now well characterised in ALS (33, 40, 121-125), clinical trials continue to exclusively focus on motor function, respiratory measures and survival as primary outcome measures. As demonstrated by this and other studies, a considerable radiological, pathological and clinical overlap exist between ALS and FTD, and motor function is just one of the facets of ALS.

There seem to be a compelling argument to also evaluate, monitor and appraise extra-motor involvement in clinical trials including executive dysfunction, memory and language (126). There is also a possibility that existing clinical trials in ALS may suffer from an inherent a selection bias to patients with no or limited cognitive impairment given the importance to comprehend, process and weighing up important information.

The involvement of Broca’s area, frontal aslant tract, inferior occipito-frontal and uncinate fascicle in bvFTD is consistent with the pathological heterogeneity of this clinical phenotype. It is also noteworthy that many of our study groups exhibit marked bilateral changes and the pathology of relatively few ROIs were limited to the left hemisphere. From an ALS perspective, our data also highlight that *C9orf72* mutations are not the sole determinants of extra-motor change. FAT RD is increased bilaterally in both ALS-FTD cohorts irrespective of *C9orf72* status and more interestingly, only the *C9orf72* negative ALS-FTD group demonstrates bilateral uncinate and right inferior longitudinal fascicle involvement. This is well in line with other ALS and ALS-FTD imaging studies where cohorts who tested negative for *C9orf72* hexanucleotide repeat also demonstrate marked cortical, suborbital and white matter pathology (25, 127, 128). Due to emerging ASO therapies, genotype-associated changes are of particular interest in both ALS and FTD (76, 105). Cluster analyses of unselected patient cohorts often revealed sub-populations of patients with particularly severe frontotemporal or cerebellar change (129-131). While *C9orf72* hexanucleotide repeats in ALS are classically associated with marked frontotemporal dysfunction, a series of studies have highlighted that extra-motor involvement is not unique to the *C9orf72* genotype (25).

This study is not without limitations. It merely assesses established language-associated tracts in a cross-sectional design. We have no post mortem data to contrast our in vivo imaging findings with post mortem pTDP-43 patterns. Similarly, no accompanying CSF or serum biomarkers were collected at the time of scanning. Notwithstanding these limitations, our results demonstrate the involvement of language associated tracts and network in patient populations along the ALS-FTD spectrum.

3.5 Conclusion

Language-associated tract and networks are not only affected in language variant FTD phenotypes but also in ALS and bvFTD. Accordingly, language deficits should be routinely screened for early in the course of the disease irrespective of *C9orf72* status. The social, management and quality of

life implications of language deficits and their potential impact on clinical trial participation should be studies in large prospective studies.

4 Chapter 3: “Motor connectome disintegration in PLS”

Full title: Not a benign motor neuron disease: longitudinal imaging captures relentless motor connectome disintegration in Primary Lateral Sclerosis

4.1 Introduction

Primary lateral sclerosis (PLS) is a low-incidence motor neuron disorder with distinctive clinical features. While the natural history and epidemiology of amyotrophic lateral sclerosis is very well characterised, disease propagation in PLS is less well understood, imaging studies are relatively scarce and post mortem studies often include a relatively small number of patients. Compared to ALS, PLS is associated with a better prognosis, and there is a prevailing assumption of slower disease progression based on clinical observations (132, 133). As there is a paucity of longitudinal neuroimaging studies in PLS, patterns of disease propagation are relatively poorly characterised and it is unclear which imaging markers are optimal for tracking cerebral disease burden. A large number of insightful cross-sectional imaging studies have been published using structural, diffusion, functional and metabolic modalities (52, 68, 74, 134-142) invariably capturing considerably primary motor cortex (PMC), corticospinal tract (CST) and corpus callosum (CC) degeneration. The PMC is typically evaluated as a single region in PLS, overlooking the predilection of symptoms to specific body regions and the likely somatotopic distribution of disease burden within the PMC. Similarly, the CST is also invariably assessed as a single tract, despite its connectivity to specific cortical regions along the motor strip. Disability in PLS is often exclusively linked to PMC and CST degeneration and with few exceptions (134) inter-hemispheric connectivity alterations are under evaluated.

Clinico-radiological correlations in other motor neuron diseases, such as ALS and SBMA are confounded by varying degree of lower motor neuron involvement and often there is a marked disparity between radiological findings and clinical disability (83, 143). As anterior horn pathology is limited or absent in PLS (144), it is an ideal condition to study clinico-radiological associations offering a rare opportunity to radiologically validate commonly used clinical instruments and cement their role in future pharmacological trials (132, 145).

Currently, there are no effective disease-modifying therapies in PLS, and despite the relatively better prognosis compared to ALS, the condition has very significant quality-of-life implications. Advances in PLS neuroimaging have been previously reviewed and a multitude of methodological approaches have been explored spanning from PET, SPECT, MRS, DTI, and a variety of structural

modalities (68, 71). The relevance of conducting large multimodal academic imaging studies is that a large panel of derived metrics can be systematically interrogated and their respective detection sensitivity and tracking potential can be juxtaposed. The practical utility of imaging markers can then be systematically appraised so that streamlined protocols can be developed for clinical and multi-site pharmacological trial applications (141). With increasing disability, patients are less likely to tolerate long imaging protocols therefore it is indispensable to establish the priority of pulse-sequences based on their biomarker utility.

Accordingly, the objective of this study is the comprehensive longitudinal analysis of motor network degeneration in PLS, using somatotopic segmentations, and body region-based connectivity analyses. Additionally, we investigate pre- and supplementary motor area involvement and contrast the sensitivity and practical utility of structural and functional connectivity metrics. Our objectives are to determine if progressive radiological changes can be detected in PLS, to verify if the interrogation of resting-state fMRI data provides additional insights, and explore the radiological correlates of routinely used clinical instruments.

4.2 Methods

Standard Protocol Approvals, Registrations, and Patient Consents

All participants gave informed consent in accordance with the Ethics Approval of this research study (Beaumont Hospital, Dublin, Ireland - IRB).

Participants and neuroimaging

Neuroimaging data from 41 PLS patients and 108 healthy controls (HC) were included, and a follow-up interval of four months was used for multi-timepoint longitudinal follow-up (**Table 4.1**). All patients had “definite PLS” according to the recently revised diagnostic criteria (146). Exclusion criteria included prior neurosurgery, previous cerebrovascular events, traumatic brain injury, comorbid neoplastic or neuroinflammatory diagnoses, comorbid psychiatric disease, and inability to tolerate MR scanning. Demographic and clinical variables were carefully recorded on the day of the scan including age, sex, handedness, medications, body region of symptom onset, family history of MND or FTD, medications, Penn Upper Motor Neuron Scale (PUMS) and its subscores, tapping rates in each extremity, and a subset of patients (n=14) were also assessed for the presence of mirror movements. The Penn UMN burden score is a composite measure of pathologically-increased reflexes in each extremity and the bulbar region, and limb spasticity measured on the modified-Ashworth scale. The number of finger and foot taps was measured over 20 seconds with 3 repetitions in each limb and the mean of the number of taps during the three trials was recorded for limb left and right. The neuropsychological profile of patients was evaluated using the Edinburgh Cognitive and Behavioural ALS Screen (ECAS), and performance was interpreted on validated population-based normative values to detect language, verbal fluency, executive, memory and visuospatial impairments. (147) Additionally, the Hospital Anxiety and Depression Scale (HADS), the Frontal Systems Behavior Scale and the Emotional Lability Questionnaire (ELQ) (148) was administered to screen for pseudobulbar affect. Exome sequencing was performed in 32 imaged PLS patients and samples screened for putative variants in the exons and splice sites of 33 genes linked to ALS (149) and 70 genes linked to HSP in the literature (150). Samples were also screened for GGGGCC hexanucleotide repeat expansion in *C9orf72* using repeat-primed polymerase chain reaction (PCR) as described previously (151).

All MR data were acquired on the same 3 Tesla Philips Achieva scanner. Structural data were acquired using a high-resolution T1-weighted (T1w) 3D Inversion Recovery Prepared Spoiled Gradient Recalled Echo (IP-SPGR) pulse sequence, diffusion-weighted data were acquired using a

spin-echo EPI sequence, and echo-planar imaging (EPI) sequence was used to evaluate blood oxygen level dependent (BOLD) signal fluctuations with the following parameters: 30 axial slices, repetition time (TR) / echo time (TE) = 2000 ms / 35 ms, flip angle (FA) = 90°, pixel bandwidth = 1780, Hz/Px. T1w and DWI parameters have been previously described (7); briefly, T1w were acquired with TR/TE = 8.5/3.9 ms, TI=1060 ms, FOV: 256 x 256 x 160 mm, and a spatial resolution: 1 mm³. 32-direction DTI images were recorded in a FOV = 245 x 245 x 150 mm, with a spatial resolution = 2.5 mm³, and TR/TE = 7639 / 59 ms. A large panel of radiological indices pertaining to motor function were systematically evaluated. Morphometric changes were investigated in cortical motor areas using cortical thickness (CT) measurements and in the medulla by volumetry. To assess the entire motor cortex, CT changes were evaluated in the primary motor cortex (PMC), premotor (PM) and supplementary motor area (SMA) using the relevant labels of the HMAT atlas (152). The Brainnetome atlas was used to define the relevant functional segments of the motor cortex (9). Both structural and functional cortex-to-medulla and PMC-to-PMC connectivity changes were explored. In each hemisphere, the integrity of three tracts was evaluated connecting the bulbar, upper and lower limb segments of the PMC with the medulla. Inter-hemispheric connectivity was evaluated separately between the contralateral PMCs.

Cortical thickness measurement and medullary volume estimates

Structural T1w data were pre-processed to extract two neuroimaging metrics, medullary volumes and the CT of five ROIs in each hemisphere: (1) “bulbar PMC”, (2) “upper limb PMC”, (3) “lower limb PMC”, (4) premotor (PM), (5) supplementary motor area (SMA). Medullary volumes were derived from FreeSurfer tools, using the “segmentBS” pipeline which segments the medulla based on Bayesian statistics (10). CT was extracted from FreeSurfer’s surface map estimating cortical thickness at each vertex. Data from all vertices comprising the given ROIs was averaged to generate a single CT value for each ROI in each subject. As surface labels are not available for the HMAT atlas, volumetric labels were mapped to the surface using Workbench tools (153). Additionally, raw T1w data were also pre-processed with FMRIB’s Software Library (FSL) pipelines to generate outputs for downstream image co-registration and normalisation to the MNI152mm space.

Functional connectivity estimation

To estimate functional connectivity (FC), data were first pre-processed in FSL utilising the FEAT pipeline. Brain extraction, slice-time correction, motion correction and correction of head-motion-

related artifacts were implemented, the latter using FSL’s AROMA algorithm (14). Each patient’s pre-processed functional image was linearly co-registered to the native high-resolution structural image using 6 degrees of freedom (DOFs), and – for higher-level group comparisons – non-linearly warped to the MNI152 2mm standard space with 12 DOFs. FC was defined as the Fisher z-transformed correlation between the mean time courses of the brainstem and the relevant cortical ROI, separately in the two hemispheres. FC was calculated in Matlab R2021b (The Mathworks, Natick, USA), using the CoSMoMVPA (15) and Fieldtrip (104) toolboxes.

Structural connectivity estimation

Structural connectivity (SC) was evaluated based on diffusion-weighted (DW) data. The data were pre-processed using MRtrix3 tools, including noise filtering (92), Gibb’s Ringing artifacts removal (18), motion & eddy current correction (19) and bias field correction (20). DW images were aligned to the high-resolution T1w data and tractograms were calculated between each pair of ROIs using a probabilistic algorithm, producing 5000 streamlines per tract (154). A more detailed description of our tractography approach has been previously reported (43). SC was defined by several metrics associated with white matter microstructure, including axial diffusivity (AD), fractional anisotropy (FA) and radial diffusivity (RD), averaged across each (binarised) tract. These were estimated based on a tensor image fitted to the data by means of weighted least squares estimation.

Statistical modelling

Statistical analyses were conducted in RStudio (R version 4.2.1). Differences in age and education between patients and controls were compared using Welch two-sample t-tests, whereas Chi-square testing was used to compare sex and handedness ratio distributions. Group differences in neuroimaging metrics were explored both cross-sectionally and longitudinally using linear models, accounting for age, sex and handedness; volumetric contrasts were also corrected for total intracranial volumes (TIV). For the cross-sectional analysis, the main effect of group membership (“PLS”) was explored and longitudinal patterns were explored in mixed effects models with the fixed effect “Subject” and the random effect “Time” using then nmle package of RStudio. This allows missing data modelling as not all subjects had follow-up data (**Table 4.1**). Since our objective was to explore progression differences between study groups, the interaction effect Group (“PLS”) x Time was evaluated. Finally, associations between neuroimaging metrics and clinical scores were assessed. For the majority of PLS patients (n=40), PUMS sub-scores lateralised to left and right and for upper and lower extremities separately as well as tapping rates were

available at one of the MR scanning session. We have therefore explored associations between clinical scores and neuroimaging using a linear model, correcting for age, sex and handedness. We limited our analysis on physiological functional associations, i.e. lateralised clinical scores with contralateral cerebral neuroimaging metrics. Only for non-lateralised bulbar PUMS scores were averaged bilateral neuroimaging metrics from both hemispheres utilised. The alpha level for all explorations was set to $p < 0.05$.

4.3 Results

Demographics

The demographic profile and statistical comparisons between study groups are presented in **Table 4.1**. The groups were adequately matched for age ($t(76.40) = 1.58$, $p = 0.12$), sex ($\chi^2(1, N = 149) = 1.03$, $p = 0.31$) and handedness ($\chi^2(1, N = 149) = 0.11$, $p = 0.74$), but PLS patients had significantly less years of education ($t(77.70) = -3.75$, $p < 0.001$). Irrespective of group differences these variables were included as covariates in the statistical models. The patients tested negative for genetic variants associated with ALS and HSP as well as GGGGCC hexanucleotide repeat expansions. Five out of 14 patients exhibited mirror movements.

Neuroimaging metrics

Statistical outcomes of neuroimaging contrasts are summarised in **Table 4.2** and illustrated in **Figures 4.1–4.4**. **Figures 4.5 and 4.6** depict the associations between neuroimaging metrics and clinical scores and **Supplementary Table 1** provides additional statistical details.

Medullary volume and motor cortex cortical thickness differentiate PLS patients from controls both at baseline and in progression

Both medullary volume and motor cortex thickness differentiate PLS from HC, both cross-sectionally and longitudinally (**Figure 4.1**). PLS patients exhibit lower medullary volumes at baseline [$t(144) = -3.72$, $p < 0.001$] and exhibit faster rates of volume loss [$t(117) = -2.43$, $p = 0.017$]. Cortical thickness of all motor cortex ROIs (bulbar PMC, upper limb PMC, lower limb PMC, PM, SMA) was significantly thinner at baseline in both hemispheres. **Figure 4.2** illustrates these findings in the left-hemisphere and **Table 4.2** provides comprehensive descriptive statistics. Moreover, in a subset of the ROIs, cortical thinning over time was more rapid in PLS; RH: bulbar ROI ($t(117) = -2.41$, $p = 0.018$), lower limb ROI ($t(117) = -2.22$, $p = 0.028$); LH: bulbar ROI ($t(117) = -$

Table 4.1 Demographic and clinical details

	PLS	HC	<i>Welch two-sample t-test [W] or Chi-squared [C2]</i>
Total number of subjects (missing T1/DWI/fMRI at baseline)	41 (1/2/3)	108 (0/1/0)	<i>n.a.</i>
Age [y, mean $\pm$ SD]	62.00 $\pm$ 10.09	59.02 $\pm$ 10.72	<i>W</i> : $t(76.40) = 1.58, p = 0.12$
Sex, F/M	16/25	54/54	<i>C2</i> : $\chi^2(1, N = 149) = 1.03, p = 0.31$
Handedness, R/L	37/4	101/7	<i>C2</i> : $\chi^2(1, N = 149) = 0.11, p = 0.74$
Baseline scans [count]	41	108	<i>n.a.</i>
4-month follow-up [count]	30	18	<i>n.a.</i>
8-month follow-up [count]	25	13	<i>n.a.</i>
12-month follow-up [count]	19	8	<i>n.a.</i>
16-month follow-up [count]	7	0	<i>n.a.</i>
Mirror movements [N=14]	5	<i>n.a.</i>	<i>n.a.</i>
Penn UMN32-score $\pm$ SD (last MRI session)	21.12 $\pm$ 6.40	<i>n.a.</i>	<i>n.a.</i>
Penn bulbar subscore $\pm$ SD (last MRI session)	2.00 $\pm$ 1.40	<i>n.a.</i>	<i>n.a.</i>
Penn R_UL subscore $\pm$ SD (last MRI session)	4.32 $\pm$ 1.70	<i>n.a.</i>	<i>n.a.</i>
Penn L_UL subscore $\pm$ SD (last MRI session)	4.41 $\pm$ 1.60	<i>n.a.</i>	<i>n.a.</i>
Penn R_LL subscore $\pm$ SD (last MRI session)	5.32 $\pm$ 1.46	<i>n.a.</i>	<i>n.a.</i>
Penn L_LL subscore $\pm$ SD (last MRI session)	5.07 $\pm$ 1.46	<i>n.a.</i>	<i>n.a.</i>
TAP20 R_UL mean $\pm$ SD (last MRI session) [N=38]	50.33 $\pm$ 22.30	<i>n.a.</i>	<i>n.a.</i>
TAP20 L_UL mean $\pm$ SD (last MRI session) [N=38]	45.34 $\pm$ 19.66	<i>n.a.</i>	<i>n.a.</i>
TAP20 R_LL mean $\pm$ SD (last MRI session) [N=38]	26.49 $\pm$ 11.70	<i>n.a.</i>	<i>n.a.</i>
TAP20 L_LL mean $\pm$ SD (last MRI session) [N=38]	25.38 $\pm$ 13.89	<i>n.a.</i>	<i>n.a.</i>
Cognitive impairment on ECAS	9 (21.9%)	<i>n.a.</i>	<i>n.a.</i>
ELQ-Laughing [N=40] mean abnormal	5.6 (31%)	<i>n.a.</i>	<i>n.a.</i>
ELQ-Crying [N=40] mean abnormal	4.0 (25%)	<i>n.a.</i>	<i>n.a.</i>

Abbreviations: ELQ = emotional lability questionnaire, F = female, HC = healthy control, L = left-handed, L_LL = left lower limb, L_UL = left upper limb, M = male, MRI = magnetic resonance imaging, N = sample size, *n.a.* = not applicable, Penn = Penn Upper Motor Neuron Score, PLS = primary lateral sclerosis, R = right-handed, R_LL = right lower limb, R_UL = right upper limb, s = seconds, SD = standard deviation, TAP20 = average number of taps in 20sec (mean), y = years, * = significant at an alpha-level of $p \leq 0.05$.

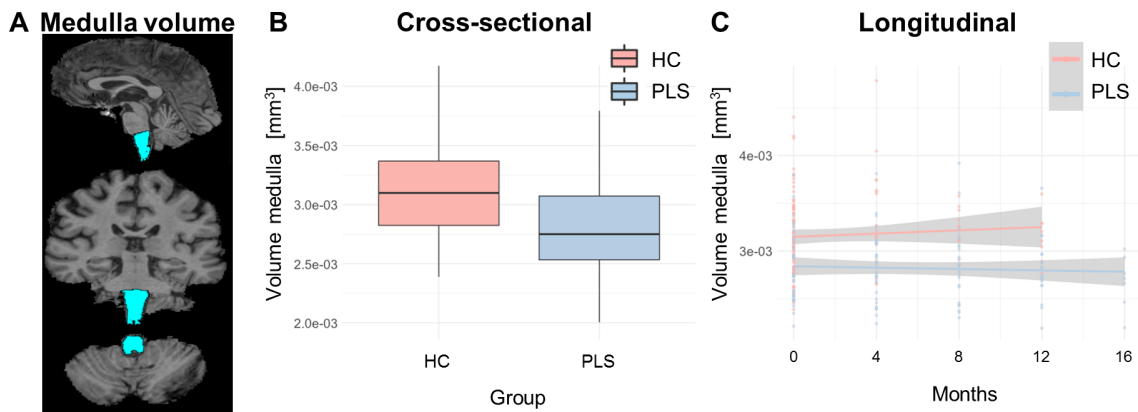


Figure 4.1 The medulla was segmented individually for each subject (A) to compare medullary volumes (MV) between patients with PLS controls cross-sectionally (B) and longitudinally (C); lower MV is observed in PLS at baseline (B) with a more rapid volume loss over time (C). Statistical details are provided in **Table 4.2**. Boxplots represent medians ± 1 quartile; whiskers denote data range spanning median $\pm 1.58 \times \text{IQR} \sqrt{n}$; dots represent outliers; IQR is the interquartile range. Longitudinal comparisons (C) are illustrated as linear models and the respective 95% confidence interval is presented by grey shading. * denotes statistical significance of unpaired t-tests, thresholded at $p \leq 0.05$. **HC**: healthy controls, **PLS**: patients with primary lateral sclerosis, **MV**: medullary volume

2.66, $p = 0.009$), upper limb ROI ($t(117) = -2.66$, $p = 0.009$), PM ($t(117) = -2.43$, $p = 0.017$)). **Table 4.2** presents the relevant statistical comparisons.

Inter-hemispheric disconnection between the motor cortices in PLS

Radiological evidence was identified for inter-hemispheric disconnection in PLS between the two motor cortices (**Figure 4.3**) as demonstrated by decreased FA [$t(144) = -2.69$, $p = 0.008$] and increased RD [$t(144) = 2.59$, $p = 0.011$] between the left and right M1. We did not detect inter-hemispheric functional PMC disconnection and no progressive changes were detected either. Output statistics are provided in **Table 4.2**.

Cortico-medullary disconnection

Our multimodal imaging data confirms reduced motor cortex - medulla connectivity in patients with PLS (**Figure 4.4**). This is evidenced by decreased FA in PLS patients at baseline in both hemispheres for the majority of relevant tracts [RH: bulbar tract ($t(144) = -2.18$, $p = 0.031$), lower limb tract ($t(144) = -3.43$, $p < 0.001$), upper limb tract ($t(144) = -2.50$, $p = 0.014$); LH: upper limb tract ($t(144) = -2.98$, $p = 0.003$), lower limb tract ($t(144) = -2.91$, $p = 0.004$)]. PM ($t(144) = -2.43$, $p = 0.017$)). A tendency for reduced functional connectivity was also detected in both hemispheres

between the bulbar ROIs and medulla [RH: $t(144) = -1.79$, $p = 0.075$; LH: $t(144) = -1.72$, $p = 0.088$]. No progressive connectivity decline was ascertained within the longitudinal follow-up interval of this cohort. **Table 4.2** summarises the relevant statistical outputs.

Associations between neuroimaging metrics are clinical scores

PUMS scores are closely associated with anatomically relevant connectivity markers (**Figure 4.5, Supplementary Table 1**): higher bulbar PUMS scores, i.e. worse disability, correlated with lower FA [$t(19) = -2.78$, $p = 0.009$], and higher RD [$t(19) = 2.58$, $p = 0.015$] in the corticobulbar tracts. Higher left arm PUMS scores correlated with lower FA [$t(19) = -2.60$, $p = 0.014$], higher AD [$t(19) = 2.67$, $p = 0.012$], and higher RD [$t(19) = 3.37$, $p = 0.002$] in the right-hemispheric “arm-CST”. Similarly, higher right

arm PUMS scores correlated with higher left-hemispheric AD [$t(19) = 3.05$, $p = 0.005$] and higher RD [$t(19) = 2.26$, $p = 0.031$]. Left leg PUMS scores correlated with higher RD [$t(19) = 2.05$, $p = 0.049$], and a tendency for higher AD [$t(19) = 1.93$, $p = 0.063$] was also detected in the right “leg-CST” i.e. the tract linking the leg-segment of the motor homunculus to the medulla. No significant associations were detected between the right lower limb PUMS scores and the relevant tracts, but a trend of association was identified between higher scores and higher RD [$t(19) = 1.77$, $p = 0.087$] and lower FC [$t(19) = -1.73$, $p = 0.100$].

Tapping rates also showed close associations with structural connectivity metrics (**Figure 4.6, Supplementary Table 1**). Lower left upper limb tapping rates correlate with lower right-hemispheric “arm-CST” FA [$t(19) = 2.62$, $p = 0.014$] and higher RD [$t(19) = -2.65$, $p = 0.013$]. Lower right hand tapping rates are associated with higher left-hemispheric “arm-CST” RD [$t(19) = -2.45$, $p = 0.021$]. Lower left foot limb tapping rates are associated with lower right-hemispheric “leg-CST” FA [$t(19) = 2.21$, $p = 0.036$] and higher RD [$t(19) = -2.24$, $p = 0.033$]. Not direct associations were detected between right lower limb tapping rates and contralateral structural connectivity, but a trend was identified for an association between lower right foot tapping rates and lower left-hemispheric FC and [$t(19) = 1.68$, $p = 0.109$]. Interestingly, no direct associations were detected between CT and PUMS score or tapping rates.

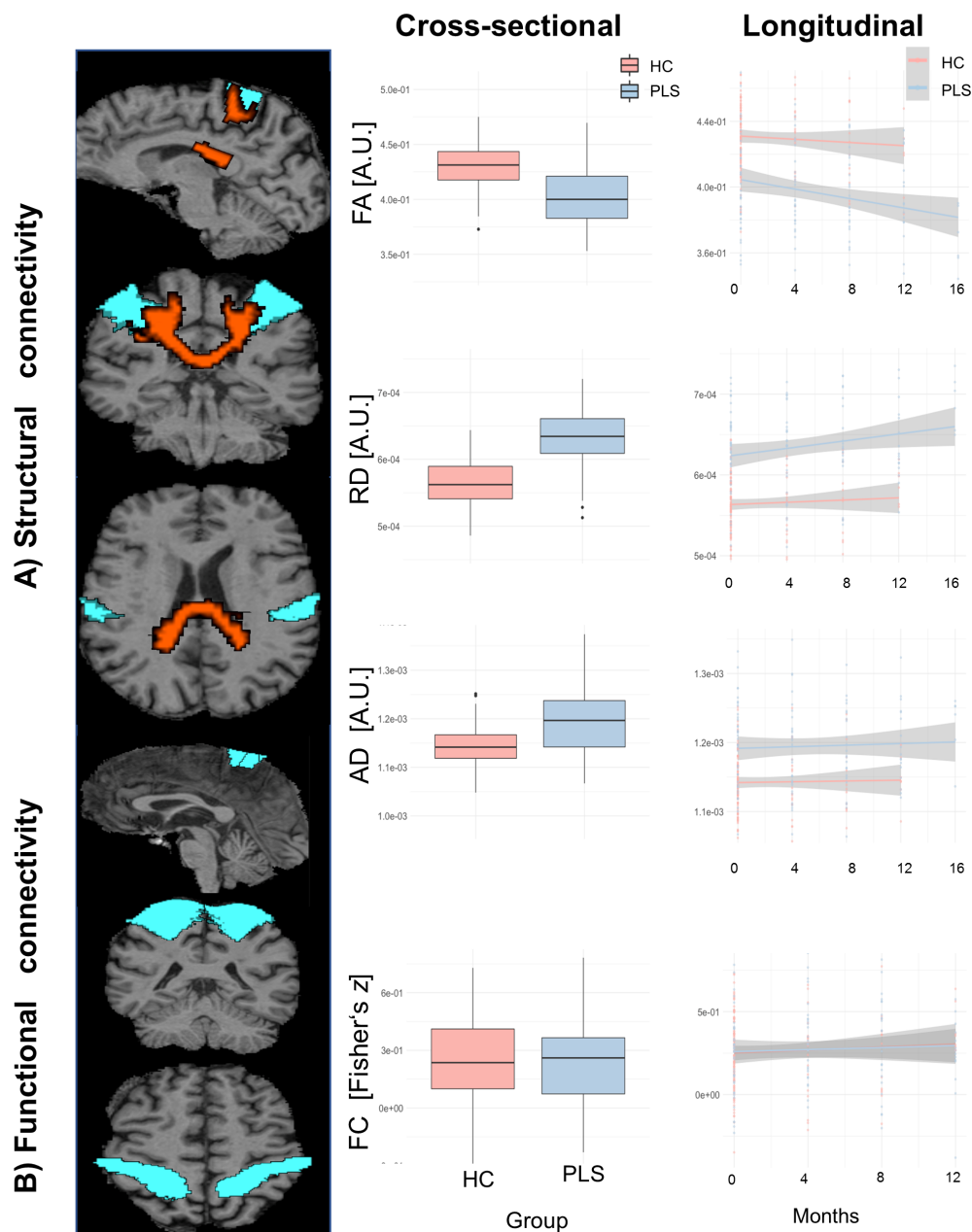


Figure 4.2 Cortical thickness (CT) of five distinct motor cortical ROIs was extracted in each hemisphere (A). At baseline (B), PLS patients exhibited reduced CT in each ROI in both hemispheres compared to controls. Similarly, the progression of cortical thinning was more rapid in PLS compared to controls in most ROIs including Bul, UL, and PM in the left hemisphere; and Bul, PM in the right hemisphere. This figure only illustrates left-hemispheric CT outcomes but **Table 4.2** provides the comprehensive statistics for both hemispheres. Boxplots (middle column) represent medians ± 1 quartile; whiskers denote the data range spanning median $\pm 1.58 \times \text{IQR} \sqrt{n}$; dots represent outliers; and IQR is the interquartile range. Longitudinal comparisons (right column) are illustrated as linear models (coloured lines) and the respective 95% confidence interval shown in grey shading. * denotes statistical significance of unpaired t-tests, thresholded at $p \leq 0.05$. **Bul**: bulbar cortex, **CT**: cortical thickness, **HC**: healthy controls, **PLS**: patients with primary lateral sclerosis, **PM**: premotor cortex, **LH**: left hemisphere, **LL**: lower limb, **RH**: right hemisphere, **ROI**: region of interest, **SMA**: supplementary motor area, **UL**: upper limb

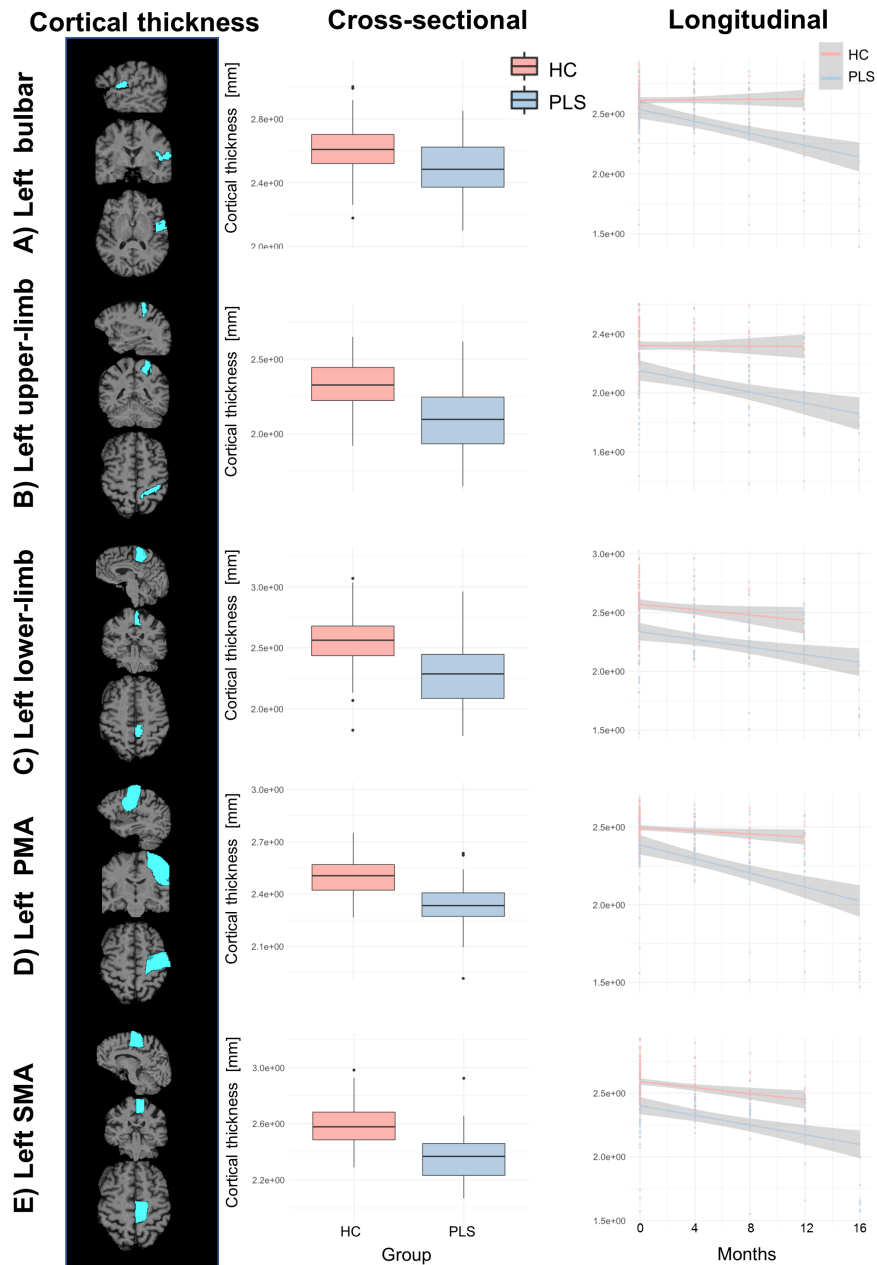


Figure 4.3 Inter-hemispheric structural (SC) and functional connectivity (FC) alterations between the contralateral primary motor cortices (A) were evaluated in patients with PLS in contrasts to healthy controls. Inter-hemispheric disconnection is suggested by significantly lower FA (B, first row) and significantly higher RD (B, second row) in PLS at baseline. No baseline AD or FC differences were identified and no longitudinal differences over time (C). **Table 4.2** provides comprehensive statistical details. Boxplots (middle column) represent medians ± 1 quartile; whiskers denote data range spanning median $\pm 1.58 \times \text{IQR} \sqrt{n}$; dots represent outliers; IQR is the interquartile range. Longitudinal comparisons (right column) are illustrated as linear models (coloured lines) and their respective 95% confidence interval shown in grey shading. * denotes statistical significance of unpaired t-tests, thresholded at $p \leq 0.05$. **AD**: axial diffusivity, **FA**: fractional anisotropy, **FC**: functional connectivity, **HC**: healthy controls, **PLS**: patients with primary lateral sclerosis, **RD**: radial diffusivity, **SC**: structural connectivity

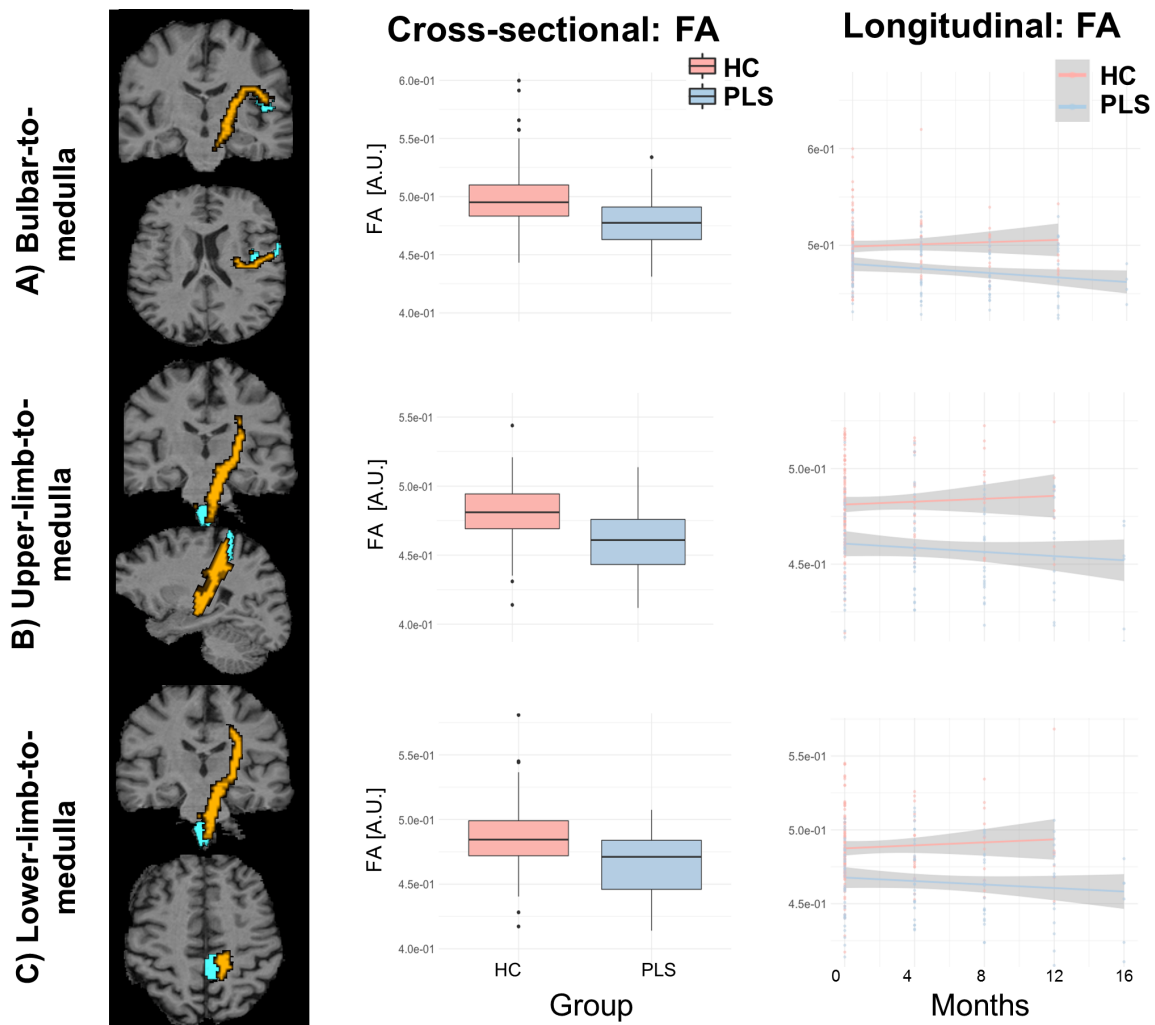


Figure 4.4 Alterations in structural cortico-medullary connectivity in PLS originating from the three functional segments of the motor strip; bulbar, upper- and lower-limb. Lower baseline FA values are detected in all three tracts in the right hemisphere and lower FA identified in the tracts originating from the upper (B) and lower-limb representation in the left hemisphere. Longitudinal progression in FA decline over the study period did not reach significance. Detailed output statistics are provided in **Table 4.2**. Boxplots (middle column) represent medians ± 1 quartile; whiskers denote data range spanning median $\pm 1.58 \times \text{IQR} \sqrt{n}$; dots represent outliers; IQR is the interquartile range. Longitudinal comparisons (right column) are illustrated as linear models (coloured lines) and their respective 95% confidence interval is shown in grey shading. * denotes statistical significance of unpaired t-tests, thresholded at $p \leq 0.05$. FA: fractional anisotropy, FC: functional connectivity, HC: healthy controls, PLS: patients with primary lateral sclerosis, ROI: region of interest, SC: structural connectivity

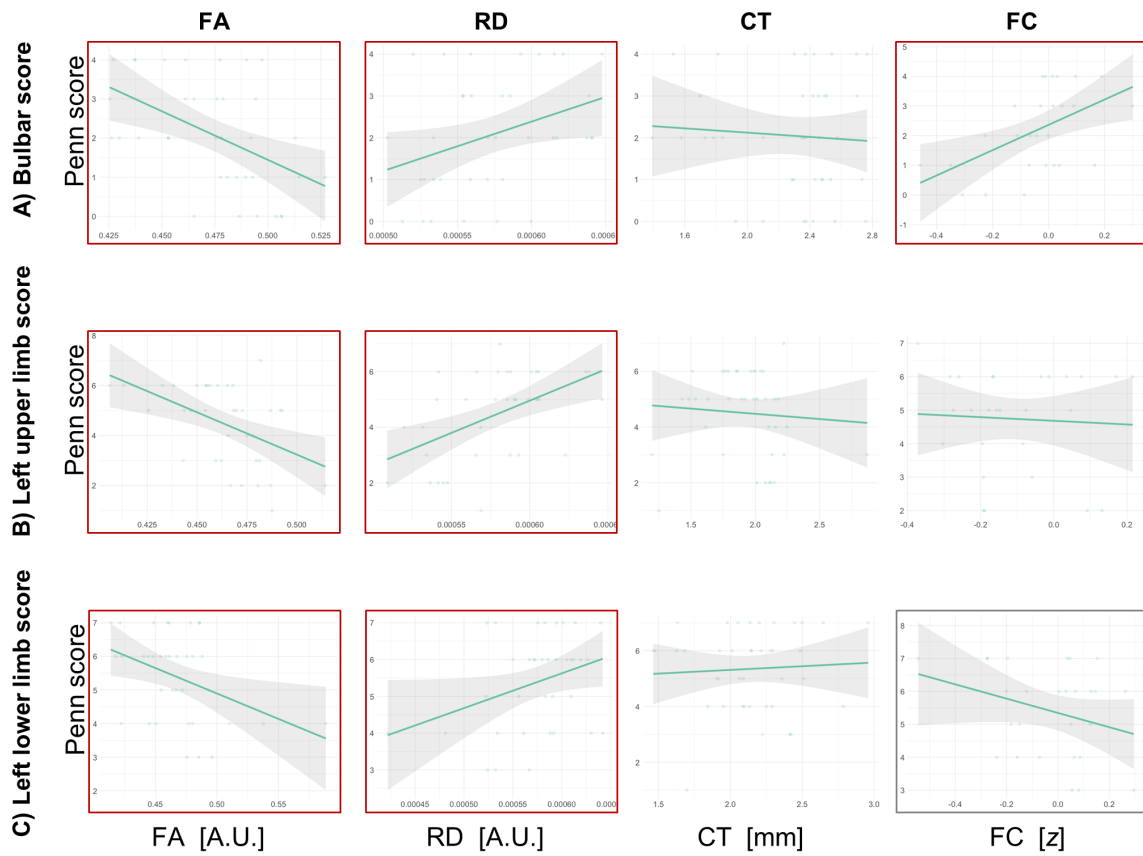


Figure 4.5 Associations between neuroimaging metrics and upper motor neuron symptom burden: Correlations between body-region-specific PUMS sub-scores and anatomically relevant MRI indices. Red boxes highlight statistically significant association at $p \leq 0.05$; grey boxes indicate a tendency for statistical significance ($p \leq .10$). Only left sided clinical scores are presented for clarity, but **Supplementary Table 1** presents detailed statistical outputs for each subscores left and right. **FA**: fractional anisotropy, **FC**: functional connectivity, **HC**: healthy controls, **PLS**: patients with primary lateral sclerosis, **PUMS**: Penn Upper Motor Neuron Score, **RD**: radial diffusivity, **ROI**: region of interest, **SC**: structural connectivity

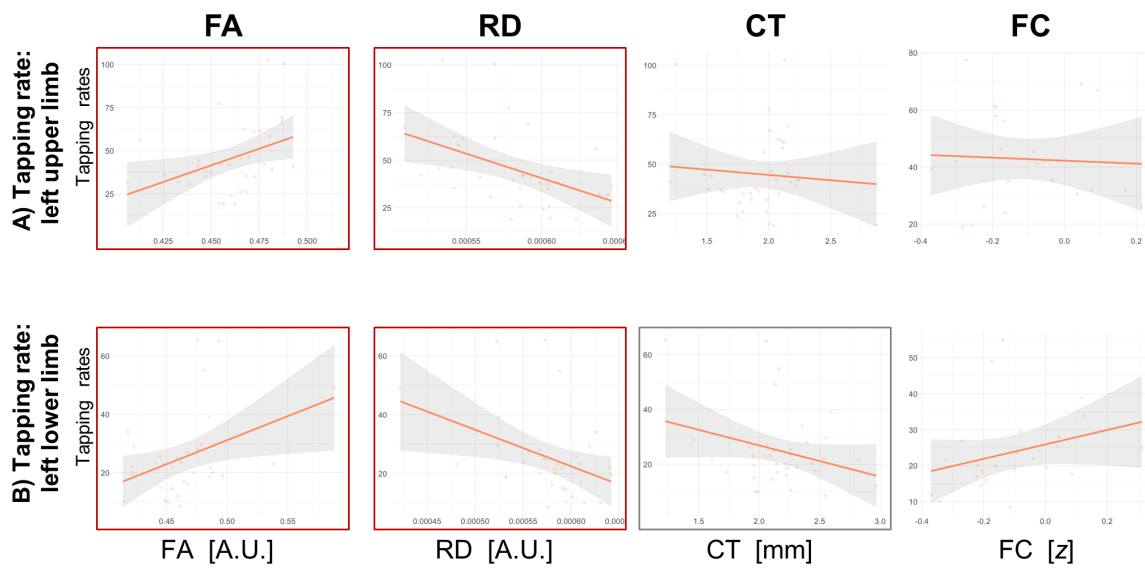


Figure 4.6 Associations between tapping rates and anatomically relevant contralateral neuroimaging metrics. Only the associations between left sided clinical scores and right hemispheric MRI measures are presented for clarity, but **Supplementary Table 1** provides detailed statistical outputs for each subscores left and right. Red boxes denote statistically significant associations at $p \leq 0.05$, grey boxes indicate a tendency for statistical significance ($p \leq .10$). **FA**: fractional anisotropy, **FC**: functional connectivity, **HC**: healthy controls, **PLS**: patients with primary lateral sclerosis, **PUMS**: Penn Upper Motor Neuron Score, **RD**: radial diffusivity, **ROI**: region of interest, **SC**: structural connectivity

Table 4.2 Statistical comparisons of neuroimaging metrics between patients with PLS and healthy controls.

	Cross-sectional (Main effect: <i>Group “PLS”</i>)						Longitudinal (Interaction: <i>Group “PLS” x Time</i>)					
	Right hemisphere			Left hemisphere			Right hemisphere			Left hemisphere		
	Esti-mate	t-value	p-value	esti-mate	t-value	p-value	esti-mate	t-value	p-value	esti-mate	t-value	p-value
<u>Medulla volume</u>												
Vol	-2.58e-4	-3.72	<.001*	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	-6.23e-5	-2.43	0.017*	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
<u>Cortical thickness</u>												
Bul	-0.162	-4.64	<.001*	-0.129	-4.08	<.001*	-0.081	-2.41	0.018*	-0.080	-2.66	0.009*
UL	-0.241	-7.07	<.001*	-0.221	-6.16	<.001*	-0.038	-1.22	0.225	-0.066	-2.66	0.009*
LL	-0.185	-4.78	<.001*	-0.274	-6.40	<.001*	-0.052	-1.87	0.064+	-0.028	-0.977	0.331
PM	-0.160	-6.21	<.001*	-0.164	-6.75	<.001*	-0.058	-2.22	0.028*	-0.062	-2.43	0.017*
SMA	-0.164	-5.27	<.001*	-0.247	-7.58	<.001*	-0.044	-1.55	0.124	-0.019	-0.670	0.504
<u>Bulbar-cortex to medulla connectivity</u>												
AD	-7.98e-6	-0.249	0.804	1.36e-5	0.328	0.743	-2.88e-6	-0.081	0.935	-9.81e-6	-0.291	0.772
FA	-0.029	-2.18	0.031*	-0.025	-1.45	0.149	-0.005	-0.411	0.682	-0.010	-0.778	0.439
RD	1.24e-5	0.792	0.430	2.96e-5	1.51	0.133	4.20e-6	0.242	0.809	3.24e-6	0.211	0.834
FC	-0.114	-1.79	0.075+	-0.068	-1.72	0.088+	-0.037	-1.40	0.165	-0.002	-0.080	0.936
<u>Upper-limb-cortex to medulla connectivity</u>												
AD	-3.79e-5	-1.04	0.302	-6.66e-6	-0.205	0.838	1.56e-5	0.456	0.649	5.99e-6	-0.178	0.859
FA	-0.049	-3.43	0.001*	-0.038	-2.98	0.003*	0.003	0.245	0.807	-0.002	-0.207	0.836
RD	7.19e-6	0.403	0.688	2.29e-5	1.42	0.157	7.11e-6	0.469	0.640	5.53e-6	0.338	0.736
FC	0.028	0.841	0.402	0.030	0.871	0.385	-0.010	-0.432	0.667	-0.021	-0.875	0.383
<u>Lower-limb-cortex to medulla connectivity</u>												
AD	-3.19e-6	-0.100	0.921	-6.28e-6	-0.194	0.846	1.08e-6	0.031	0.975	-6.16e-6	-0.174	0.862
FA	-0.032	-2.50	0.014*	-0.038	-2.91	0.004*	-0.003	-0.234	0.815	-0.007	0.583	0.561

Chapter 3: “Motor connectome disintegration in PLS”

RD	1.89e-5	1.20	0.230	2.28e-5	1.42	0.158	3.10e-6	0.188	0.852	1.17e-6	0.074	0.942
FC	0.019	0.617	0.538	0.0513	1.60	0.113	-0.014	-0.662	0.5091	-0.019	-0.800	0.426
Inter-PMC connectivity												
AD	2.69e-5	0.737	0.462	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	-7.71e-6	-0.227	0.821	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
FA	-0.035	-2.69	0.008*	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	-0.007	-0.695	0.489	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
RD	5.05e-5	2.59	0.011*	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	4.16e-7	0.025	0.980	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
FC	-0.040	-0.893	0.374	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	0.009	0.262	0.794	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>

Bilateral metrics are presented in under the relevant hemisphere; metrics from single structures such as medullary volumes and inter-hemispheric connectivity metrics are presented in the “Right hemisphere” columns.

Abbreviations: AD = axial diffusivity, Bul = bulbar, CT = cortical thickness, FA = fractional anisotropy, FC = functional connectivity, HC = healthy control, LL = lower limb, PMC = primary motor cortex, MRI = magnetic resonance imaging, *n.a.* = not applicable, PM = premotor cortex, RD = radial diffusivity, SMA = supplementary motor area, TAP = tapping, UL = upper limb, Vol = volumetry (of the medulla), * = significant at an alpha-level of $p \leq 0.05$, + = approaching significance at an alpha-level of $p \leq$

4.4 Discussion

In addition to confirming progressive primary motor cortex degeneration, our results also demonstrate considerable supplementary- and pre-motor involvement, progressive brainstem atrophy, cortico-medullary and inter-hemispheric disconnection, and close associations between clinical UMN scores and somatotopic connectivity indices.

Motor disability in PLS is typically exclusively linked to primary motor cortex degeneration, but our analyses demonstrate additional pre- and supplementary- motor area pathology which are likely to contribute to motor disability. The premotor area is classically implicated in movement planning, the preparation of postural muscles for forthcoming movements, spatial guidance of movement, and in the sensory guidance of movements. SMA is thought to contribute to postural stability, movement initiation, coordination of temporal sequences of actions, and bimanual coordination. Similar to ALS, extrapyramidal dysfunction is also likely to significantly contribute to motor disability in PLS (117, 118, 123, 155). Previous PLS studies have invariably evaluated the primary motor cortex as a single cortical region without its somatotopic segmentation. Similarly, previous PLS studies evaluate the corticospinal tract as a single tract, without parcellation into sub-tracts originating from the lower limb, upper limb and bulbar segments of the motor cortex. The segmentation of the PMC and CST into functionally defined, somatotopic sub-regions permits a more nuanced characterisation of motor connectome degeneration. In turn, the interrogation of the integrity metrics of these labels allows more direct associations with regional UMN metrics. In other MNDs, such as ALS, direct clinico-radiological correlations between disability scores and cerebral measures are widely considered contentious (83) as motor disability stems from a combination of upper- and lower motor neuron pathology often disproportionately driven by LMN dysfunction. PLS is an ideal phenotype to explore associations between brain metrics and clinical measures which in turn permits both the validation of clinical instruments as well as highlighting the value of MRI-derived indices (145, 156).

The clinico-radiological correlations of this study corroborate the validity and utility of cheap, easily-administered clinical tests, both established instruments (PUMNS) as well as simple quantitative tools such as tapping rates. Tapping rates exhibit excellent concordance with contralateral CST metrics both in the upper- and lower limb segments. These associations suggest that disability is closely related to motor connectome disintegration and suggest that these clinical tests could be readily used for both monitoring purposes as well as potential endpoints in clinical trials. The lack of direct associations between cortical thickness measures and clinical UMN signs confirms the value of connectivity metrics. It also highlights that clinical disability should not be

linked to focal cortical measures alone, but symptoms stem from the disconnection of physiologically interconnected brain regions, in this case, the close interplay between the brainstem, motor cortex, pre- and supplementary motor regions.

Our findings reveal that disability in PLS is primarily driven by somatotopic degeneration of the motor system, and PLS is not solely associated with intra-hemispheric but also inter-hemispheric degeneration disconnection. While the evaluation of FA is sometimes criticised as a mere composite measure of white matter integrity, it has captured baseline connectivity changes where AD and RD did not detect significant alterations. RD was also affected in the inter-hemispheric callosal fibres connecting the contralateral motor cortices. Despite the limited RD and AD changes in our comparative analyses where PLS patients were contrasted to controls, these measures showed excellent correlation with clinical metrics highlighting the importance of evaluating a large panel of white matter metrics rather than FA alone.

The relatively limited longitudinal change in connectivity metrics over the 16 month follow-up suggest that considerable change may have already taken place at study entry and that rate of progression after diagnosis may be relatively slow; this is sometimes referred to as ceiling- or flooring-effect of a given variable. These metrics may have a good utility in differentiating patients from controls, but limited in tracking patients over time. Conversely, medullary volumes and cortical thickness values in the bulbar segment of the motor cortex and the premotor area continue to decline over time, making them better suited for monitoring markers. From a biomarker perspective, no single imaging marker can address both the diagnostic and monitoring requirements of PLS and therefore, a panel of several metrics with complementary detection profiles are required. One of the practical deliverables of academic imaging studies is the appraisal of a large panel of imaging measures with regards to their clinical utility so that streamlined, short-duration protocols can be developed for the pragmatic needs of clinical and pharmacological trial applications. Accordingly, our observations may have implications for the design of clinical protocols. While changes in fMRI connectivity metrics are largely consistent with diffusivity-derived structural connectivity alterations, they are less significant and add relatively little additional information. This is further demonstrated by our inter-hemispheric analyses where FA, AD, RD readily capture the disconnection between the two PMCs whereas fMRI fails to detect this. It is also noteworthy that raw fMRI data typically has a much lower spatial resolution than DTI data, and pre-processing and subsequent data analyses are also often more complex (39). Furthermore, the impact of underlying vascular and pharmacological factors, such as hypertension, smoking, diabetes, and medications such as TCAs, SSRIS, benzodiazepines and

central acting anti-spasticity medications on BOLD signal are not fully elucidated and may be important confounders. Accordingly, our data suggest that in clinical applications, a high-resolution 3D T1-weighted pulse sequence and a DTI sequence offers ample metrics to quantitatively assess grey and white matter integrity and the practical biomarker value of routine rs-fMRI acquisition may be relatively limited. Other imaging modalities, such as high angular resolution diffusion-weighted imaging (HARDI), neurite orientation dispersion and density imaging (NODDI) or magnetic resonance spectroscopy (MRS) are complementary techniques which have generated important insights in other motor neuron diseases (37). The additional relevance of evaluating a large panel of metrics is gauging their potential as features in future machine-learning (ML) applications. ML models are increasingly applied to MND data sets and a variety of mathematic models have been recently explored (51, 157).

This paper is not without limitations, mirror movements were only evaluated in 14 patients and genetic information is only available in 32 patients. fMRI analyses are limited to resting-state data as no motor paradigms were utilised in this study. All of the participants had “definite” PLS based on the recent consensus criteria consistent with their relatively long symptom duration. The inclusion of additional patients with “probable” PLS may have generated additional insights with regards to the earlier phases of PLS (72, 158). Notwithstanding these limitations, our paper demonstrates progressive motor connectome disconnection in PLS, significant inter-hemispheric connectivity alterations, somatotopic involvement of cortical regions and descending corticospinal tracts. The considerable association with simple, cheap and readily administered clinical metrics highlight the utility of these instruments in everyday clinical practice.

4.5 Conclusions

PLS is associated with both intra- and inter-hemispheric connectivity reductions. Simple clinical instruments, such as UMN scores and tapping rates readily correlate with MRI-derived pyramidal tract measures confirming their utility in assessing disease burden non-invasively.

5 Chapter 4: “Pseudobulbar affect in PLS”

Full title: Radiological correlates of pseudobulbar affect: corticobulbar and cerebellar components in Primary Lateral Sclerosis

5.1 Introduction

Pseudobulbar affect (PBA) refers to recurrent episodes of exaggerated emotional reaction to minimal external stimulus which would have not typically triggered such as response in the affected individual previously. Context-inappropriate crying or laughing are the most commonly reported symptoms, but excessive yawning is also considered to be part of the clinical spectrum of PBA. Commonly used alternative terminology includes “pathological crying and laughing (PCL)”, “emotional lability (EL)”, or “involuntary emotional expression disorder (IEED)” (159, 160). The sudden outbursts associated with PBA are often in line with the individual’s transient emotional state (62, 161, 162), but the responses are invariably disproportionate to the stimulus and often inappropriate to the social context.

PBA has been observed in a multitude of neurological syndromes spanning from acquired brain injury to neurodegenerative conditions, but it is particularly common in motor neuron diseases (MND), multiple sclerosis (MS), stroke, multiple system-atrophy-cerebellar type (MSA-C), traumatic brain injury (TBI), Alzheimer’s disease, and Parkinson’s disease. Irrespective of the underlying aetiology, PBA has significant quality of life implications. Patients with PBA typically find the unprovoked outbursts of emotional responses very troublesome, and often chose to limit social interactions due the fear their symptoms may be misinterpreted as psychiatric or cognitive manifestations. PBA can cause considerable embarrassment, social withdrawal and has a multitude of quality of life implications. (163)

Albeit PBA has a rich and well documented literature, no single unifying pathophysiological explanation exists. While the classical view (164) on the underlying mechanism centres on impaired corticobulbar control of ingrained brainstem responses, the contribution of impaired cerebellar gaiting processes, serotonergic and frontotemporal dysfunction are increasingly recognised (165) (59-62, 166-168). It may be the case that several mechanisms converge to the unique constellation of clinical symptoms referred to as PBA or that divergent aetiologies may lead to PBA in different conditions. Within the MND spectrum PBA is most commonly reported in patients with primary lateral sclerosis (PLS) or upper motor neuron-predominant

amyotrophic lateral sclerosis (ALS) and not commonly observed in lower motor neuron predominant syndromes such as Kennedy’s disease, progressive muscular atrophy (PMA) or adult spinal-muscular atrophy (63, 69, 143, 169-175). The study of clinically well-characterised homogenous diagnostic groups may offer unique opportunities to evaluate the neuronal underpinnings of PBA. The most widely accepted pathomechanism of PBA is brainstem-cortex disconnection, but in motor neuron diseases, cerebellar processes have also been consistently implicated (57, 59-62). Frontotemporal dysfunction is well characterised in ALS (67, 74, 176) and frontal lobe atrophy (59) and fronto-cerebellar connectivity alterations have been specifically linked to PBA (61).

Accordingly the overarching objective of this study is the nuanced evaluation of the radiological correlates of PBA without anatomical *a priori* hypotheses in a large cohort of clinically- and genetically well-characterised cohort of primary lateral sclerosis patients. Based on data from ALS (59, 61, 62), an additional objective of our study is the targeted assessment of the two most commonly proposed pathomechanisms, namely the contribution of corticobulbar and cerebellar disease-burden to PBA.

5.2 Methods

Standard Protocol Approvals, Registrations, and Patient Consents

All participants gave informed consent in accordance with the Ethics Approval of this research study (Beaumont Hospital, Dublin, Ireland - IRB).

Participants

In a single-centre, prospective neuroimaging study, multimodal MR data from 35 patients with primary lateral sclerosis were systematically analysed (**Table 5.1**). Patients were diagnosed in accordance with the current consensus diagnostic criteria (146). Exclusion criteria included prior neurosurgery, antecedent cerebrovascular events, preceding traumatic brain injury, established neoplastic, neuroinflammatory or psychiatric conditions. Comorbid frontotemporal dementia was not considered an exclusion criterion. Patients were screened for neuropsychological deficits and depression using the Edinburgh Cognitive and Behavioural ALS Screen (ECAS) (177), the Frontal Systems Behavior Scale (FrSBe), and the Hospital Anxiety and Depression Scale (HADS). The Emotional Lability Questionnaire (ELQ) was administered to evaluate the severity of PBA (148) and this score was used to explore the anatomical correlates of PBA. The ELQ is a 33-item questionnaire divided into three main domains; laughing, crying and smiling. Motor disability was appraised using the revised ALS functional rating scale (ALSFRS-r) which offers a composite score of disability in four main domains; bulbar, upper-limb, lower-limb and respiratory function (178). The Edinburgh Cognitive and Behavioural Screen (ECAS) screens for cognitive impairment in language, verbal fluency, executive, memory and visuospatial domains (177) and has been thoroughly validated in the Irish population with the generation of local normative data (147). The FrSBe evaluates apathy, disinhibition and executive dysfunction across 46 items (179).

Genetics

All participating patients underwent whole exome sequencing, as previously described (63). Protein altering variants in the exons and splice sites of 33 genes associated with ALS on the ALS online database (149) and 70 genes associated with hereditary spastic paraplegia (HSP) in the literature (150) were considered putative genetic variants. *C9orf72* hexanucleotide repeat expansion (HRE) status was evaluated using repeat-primed polymerase chain reaction (PCR) as described previously (151).

Table 5.1 Demographic and clinical details of the PLS study population.

	PLS patients
Age (year) - mean $\pm$ SD]	61.06 $\pm$ 9.77
Sex - F / M	15 / 20
Handedness - R / L	31 / 4
Years of education - mean $\pm$ SD	12.97 $\pm$ 3.16
ELQ score - mean $\pm$ SD	12.34 $\pm$ 14.91
ELQ score - median, IQR	5 [0.17; 24]
Symptom duration (months) - mean $\pm$ SD	91.6 $\pm$ 18.7
ALSFRS-r - mean $\pm$ SD	33.88 $\pm$ 5.05
Bulbar subscore (max 12) - mean $\pm$ SD	9.25 $\pm$ 2.24
ECAS - abnormal n (%)	16 (45.71%)
ECAS - mean $\pm$ SD	104.25 $\pm$ 18.57
FrSBe - abnormal n (%)	10 (28.57%)
HADS mean $\pm$ SD	8.25 (4.67)
Missing MRI data for volumetry / CTh / CERES / DWI / rs-fMRI	0 / 2 / 10 / 0 / 11

Abbreviations: CERES = CEREBellum Segmentation, CTh = cortical thickness, DWI = diffusion weighted imaging, ECAS = Edinburgh Cognitive and Behavioural ALS Screen, ELQ = emotional lability questionnaire, F = female, FrSBe = Frontal Systems Behavior Scale, HADS = Hospital Anxiety and Depression Scale, IQR = interquartile range, L = left-handed, M = male, PLS = primary lateral sclerosis, R = right(-handed), rs-fMRI = resting state functional magnetic resonance imaging, SD = standard deviation, y = years

Study design

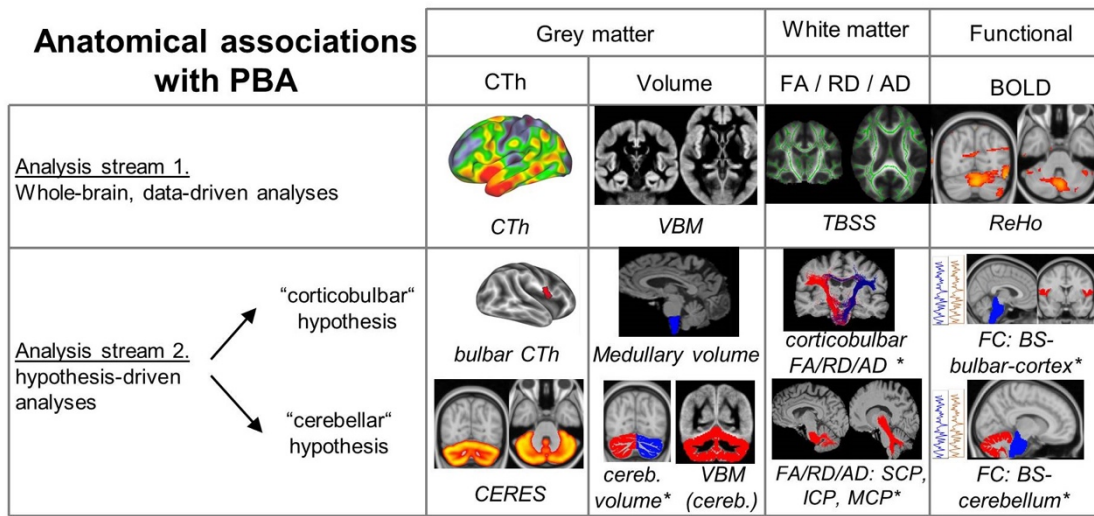
The main objective of the study was to investigate the neuroimaging correlates of the pseudobulbar affect (PBA) as measured by the emotional lability questionnaire (148). Multiple MR sequences and their respective derived metrics were analysed: 1) structural T1-weighted (T1w) data to estimate grey matter (GM) cortical thickness and volumes 2) diffusion-weighted imaging (DWI) to evaluate white matter (WM) microstructure integrity and 3) blood-oxygen-level-dependent (BOLD) signal acquired from resting-state functional MR imaging to assess functional connectivity (FC).

Two main analysis streams were pursued (**Figure 5.1**); (1) a data-driven whole-brain exploratory arm without *a priori* anatomical hypotheses and (2) region-of-interest strategy

based on prevailing pathophysiological theories. We started with the exploratory approach, i.e. we tested correlations between PBA scores and whole-brain data with no anatomical restrictions. The advantage of this approach is the potential to identify anatomical associations in an unbiased manner, which comes at the cost of reduced power given the increased numbers of statistical comparisons and thus elevated family-wise error (FWE) rate. While a variety of validated methods are available to control for FWE rate in neuroimaging, such as threshold-free cluster enhancement (TFCE) (180), we have also reviewed non-FWE-corrected outputs. In the second analysis stream, we followed a hypothesis-driven approach. In line with the two most common pathophysiological conceptualisation of PBA, the “corticobulbar bulbar hypothesis” and “cerebellar hypothesis”, cortico-bulbar connectivity and cerebellar connectivity were specifically evaluated based on both structural and functional data. In each analysis stream, grey matter (GM), white matter (WM) and functional correlates of PBA were explored.

Data acquisition

All imaging data were uniformly acquired on the same 3 Tesla Philips Achieva scanner with an eight-channel receiver-only head coil. Three different MR pulse sequences were implemented to assess tissue types and connectivity. T1-weighted data were acquired with a 3D inversion recovery-prepared spoiled gradient recalled echo (IR-SPGR) sequence with the following settings: TR/TE = 8.5/3.9 ms, TI = 1060 ms, matrix size = 256 × 256 mm, 160 sagittal slices, spatial resolution: 1 mm isotropic, flip angle, (FA) = 8° and SENSE factor = 1.5. 2) Diffusion-weighted data were acquired with a spin-echo planar imaging (SE-EPI) pulse sequence with a 32-direction Stejskal-Tanner diffusion encoding scheme (b-values = 0 / 1100 s/mm²) with the following main parameters: TR/TE = 7639 / 59 ms, matrix size = 245 × 245, 60 axial slices with no interslice gaps, spatial resolution = 2.5 mm isotropic, FA = 90°, SENSE factor = 2.5, dynamic stabilisation and spectral presaturation with inversion recovery (SPIR) fat suppression. Resting-state functional imaging (rsMRI) data were recorded with a T2-weighted EPI sequence to investigate BOLD signal fluctuations. 220 volumes were recorded with eyes closed implementing the following parameters: TR/TE = 2000 ms / 35 ms, matrix size = 233 x 233, 30 axial slices, spatial resolution = 2.875 x 2.875 x 4 mm³, flip angle (FA) = 90°, SENSE factor = 2.5, pixel bandwidth ~ 1900, Hz/Px (with slight variations of up to 150 Hz/Px between subjects). For



* separately for the left / right hemispheres

Figure 5.1 The flowchart of the study design. Associations between pseudobulbar affect and multimodal neuroimaging metrics were analysed two main analysis streams (1) A data-driven, whole-brain, exploratory study without *a priori* anatomical hypotheses and (2) region-of-interest pipeline testing the “corticobulbar” and “cerebellar” theories of PBA. For each analysis streams, a panel of grey matter, white matter and functional imaging metrics were evaluated.

Abbreviations: AD = axial diffusivity, BOLD = blood-oxygen-level-dependent, BS = brainstem, cereb. = cerebellum, CERES = CEREBellar Segmentation, CTh = cortical thickness, FC = functional connectivity, FA = fractional anisotropy, ICP = inferior cerebellar peduncle, MCP = middle cerebellar peduncle, PBA = pseudobulbar affect, RD = radial diffusivity, ReHo = regional homogeneity, SCP = superior cerebellar peduncle, TBSS = tract-based spatial statistics, VBM = voxel-based morphometry

both analysis streams (Whole-brain and ROI), we retrieved GM, WM and BOLD neuroimaging metrics to evaluate anatomical associations with PBA scores.

We describe the operationalisation and calculation of these metrics below, stratified by the analysis streams (**Figure 5.1**). Relevant demographic variables including age, sex, handedness and years of education were recorded on the day of scanning to correct for potential confounds (**Table 5.1**). Relevant clinical data including the ECAS, HADS, ALSFRS-r, ELQ were collected within 2 weeks of the scan (148, 177, 178)

Analysis stream 1: Whole-brain analyses

To explore whole-brain associations between grey matter morphometry and PBA, structural T1w data were pre-processed to derive estimates on both cortical thickness (CTh) and volumes. Whole-brain CTh maps were calculated using FreeSurfer’s (version 7.3.2) automated pre-processing pipeline “recon-all” (8), which generates surface maps indicating CTh at each

vertex. These data were converted into the CIFTI file format using the Ciftify toolbox to facilitate post-processing and visualization (86). To explore GM alterations beyond CTh, voxel-based morphometry (VBM) was also performed using FMRIB’s Software Library (FSL) version 6.0 (12) implementing the standard pipeline (19, 181, 182). Associations between WM microstructure integrity metrics and PBA were investigated over the entire brain using FSL’s tract-based spatial statistics (TBSS) (183). DWI data were pre-processed within MRtrix3 (version 3.0.3) (16) to generate individual fractional anisotropy (FA), radial diffusivity (RD) and axial diffusivity (AD) maps as described previously (43, 184). In brief, pre-processing included de-noising (17, 92), removal of Gibb’s Ringing Artifacts (18), motion and eddy current correction (19) and bias field correction (20). The pre-processed DW images were subsequently aligned to the high-resolution T1-weighted data. Resulting FA, RD and AD maps were fed into FSL’s standard TBSS pipeline with registration to the FMRIB58-FA standard-space image, and nonlinear transformation to all subjects’ FA, RD and AD images (183). Whole-brain associations between BOLD signal fluctuations and PBA scores were explored as variations of regional homogeneity (ReHo) (185, 186). In brief, ReHo is a measure of functional network centrality and reflects synchronization of neural activity i.e. abnormal ReHo is regarded as proxy of altered neuronal activity. For example, increased ReHo may suggest excessive activity of local neuronal populations, increased internal consistency and decreased synchronicity with other brain regions (187). FSL’s FEAT pipeline was used to pre-process BOLD data for ReHo including brain extraction, slice-time correction, and motion correction. The first five volumes were discarded to account for signal equilibrium. Correction of head-motion-related artifacts was added in a separate data preparation step using FSL’s “AROMA” algorithm (14). ReHo maps were computed within the Analysis of Functional NeuroImages (AFNI) suite (version 23.0.07) (188, 189), using the “3dReHo” algorithm, which outputs voxel-wise maps of Kendall’s W (190). Correlations between the above whole-brain neuroimaging metrics and PBA scores (corrected for age, sex, handedness and years of education) were evaluated using FSL’s “randomise” tool with 5000 permutations (23). Associations between higher PBA scores and decreased radiological integrity i.e. decreased CTh, VBM-T1 signal, FA and increased AD, RD and ReHo were specifically investigated. We have subsequently reviewed both TFCE-corrected and uncorrected statistical maps evaluated at an alpha-threshold of $p \leq 0.05$.

Analysis stream 2: hypothesis-driven Corticobulbar and cerebellar associations

The “corticobulbar” and “cerebellar” hypotheses were evaluated separately. The bulbar portion of the primary motor cortex was defined based on label “A4tl” of the Brainnetome atlas (9). The medulla was first segmented using FreeSurfer’s segmentBS tool (10). We investigated GM-PBA associations based on medullary volumes and cortical thickness in the bulbar portion of the motor homunculus separately in right and left hemispheres using Matlab R2022b (The Mathworks, Natick, USA), tools from Workbench (153) and CoSMoMVPA (15). GM variations in the cerebellum were operationalised as 1) the volume of the cerebellar hemispheres as per the “recon-all” output of the FreeSurfer pipeline, 2) VBM as described above, but restricted to a binary cerebellar mask and 3) cerebellar CTh maps using the software CEREBellar Segmentation, “CERES” (191), within volBrain (192). CERES is a well-validated structural segmentation pipeline of the human cerebellum (193) which reliably generates voxelwise maps of cerebellar CTh for subsequent statistical interpretation.

WM-PBA associations for the “corticobulbar hypothesis” were operationalised as FA, RD and AD of the corticobulbar tract. The corticobulbar tract was defined as tracts linking the medulla and to the bulbar segment of the primary motor cortex (**Figure 5.2**), and accordingly, a fixed number ($N = 5000$) of corticobulbar streamlines were tracked between those two brain regions in MRtrix3. Tracking was performed separately for each hemisphere in each individual in native space. We estimated fibre orientation distributions (fODF) using the constrained spherical deconvolution model (CSD)(97, 98) and estimated streamlines based on a probabilistic algorithm (154). We then binarised these tractograms to extract the mean FA, RD and AD values per tract and subject using the previously calculated tensor maps. Similarly, WM-PBA associations for the “cerebellar hypothesis” were also operationalized as FA, RD and AD alterations in the five cerebellar peduncle tracts, the left (Lt) and right (Rt) superior cerebellar peduncles (SCP), the middle cerebellar peduncle (MCP) and left and right inferior cerebellar peduncles (ICP) (**Figure 5.2**). To identify these tracts, we used the TractSeg segmentation pipeline (96, 194), which relies on a neural network algorithm to segment the WM tracts. TractSeg allows to estimate fODF using MRtrix3’s CSD model, so that we could estimate cerebellar tracts analogously to the bulbar tract as outlined above. We binarised the cerebellar tracts and extracted mean FA, RD and AD values along the course of each tract.

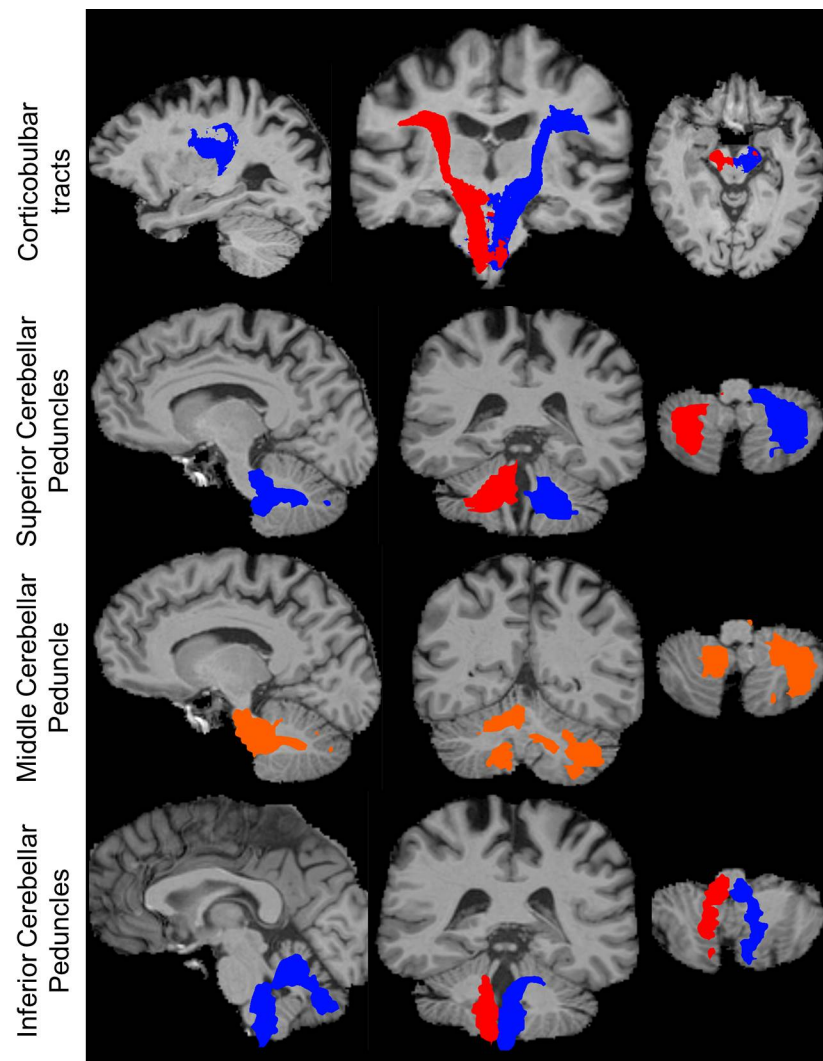


Figure 5.2 Tractography for the corticobulbar tracts and cerebellar peduncles Representative sagittal, coronal and axial views are shown (left to right)

Finally, BOLD-PBA associations were investigated based on functional connectivity (FC) between (1) the brainstem and the bulbar segment of the primary motor cortex to assess the “corticiobulbar hypothesis” and (2) between the brainstem and the cerebellum to test the “cerebellar hypothesis” separately in the left and right hemispheres. FC was calculated using the pre-processed BOLD data, extracting the mean time series of all voxels comprising the respective ROIs and computing pairwise Pearson’s correlations between the pairwise ROIs within Matlab R 2022b.

Statistical associations between PBA scores and cerebellar VBM and CERES were investigated voxelwise using the relevant neuroimaging maps and design matrices, analogous to analysis stream 1, as described above. For the assessment of scalar metrics, such as ROI-wise volumes, CTh, FA, RD, AD and FC), we used a linear model, in which we modelled the specific neuroimaging metric as the independent and PBA scores as the dependent variable, accounting for the potential confounding effect of age, sex, handedness and years of education. These analyses were run within RStudio version 2023.03.0, using R Version 4.3.0 (195).

For the anatomical labelling of the relevant brain regions, we used the grey matter labels of the Harvard-Oxford cortical and subcortical structural atlases (196) and the MNI Cerebellar Atlas (197) and for the white matter, the JHU (198) and the XTRACT (199) atlases were utilised.

5.3 Results

Demographics

The demographic and clinical details of the study cohort are summarised in **Table 5.1**. As not every imaging modality was available for every patient, missing data were excluded from the respective analysis streams. All patients tested negative for *C9orf72* HRE as well as the panel of ALS and HSP associated genetic variants. ELQ scores did not show associations with ALSFRS-r ($p = .920117$), HADS-A, ($p = .47726$) HADS-D ($p = .8494$) or HADS-Total ($p = .64463$). The results of the correlation analyses between PBA and neuroimaging metrics are illustrated in **Figures 5.3–5.4**, spanning from data-driven to hypothesis-driven analyses. Comprehensive statistical details are provided in **Table 5.2** and **Table 5.3**.

Whole-brain analysis

In the first analysis stream, GM, WM and functional signal changes were considered over the entire brain in relation to PBA. Following TFCE-corrections, we found evidence that PBA is associated with WM changes in multiple anatomical regions (**Figure 5.3A**). FA reductions correlated to increased ELQ scores in the corpus callosum (CC) and bilateral corticospinal tracts (CST), as well as in some association tracts. Increased RD was associated with higher ELQ scores, but only evident in the body of the CC. No significant associations were identified on AD TBSS analyses. For GM (as measured by VBM and CTh) and BOLD (as measured by ReHo) analyses, no voxels survived TFCE-corrections. However, given the exploratory nature of this first analysis stream, uncorrected p -maps were also reviewed thresholded at $p_{\text{uncorr}} \leq 0.05$, for both VBM (**Figure 5.3B**) and ReHo (**Figure 5.3C**) analyses. On these uncorrected maps, reduced T1w signal on VBM was associated with higher ELQ scores in the cerebellum and some frontal cortical regions. Increased ReHo in the brainstem was associated with increased ELQ scores. No associations were captured between PBA and CTh on whole-brain analyses, even when uncorrected p -maps were reviewed.

“Corticobulbar hypothesis”: Associations with structural and functional corticobulbar connectivity

The whole-brain ReHo, VBM and TBSS findings were supportive of the “corticobulbar hypothesis”, therefore this was specifically tested in dedicated ROI analyses. **Figure 5.4** summarises the significant results of this analysis stream and comprehensive statistical details are provided in **Table 5.2** The pathology of the right-hemispheric corticobulbar

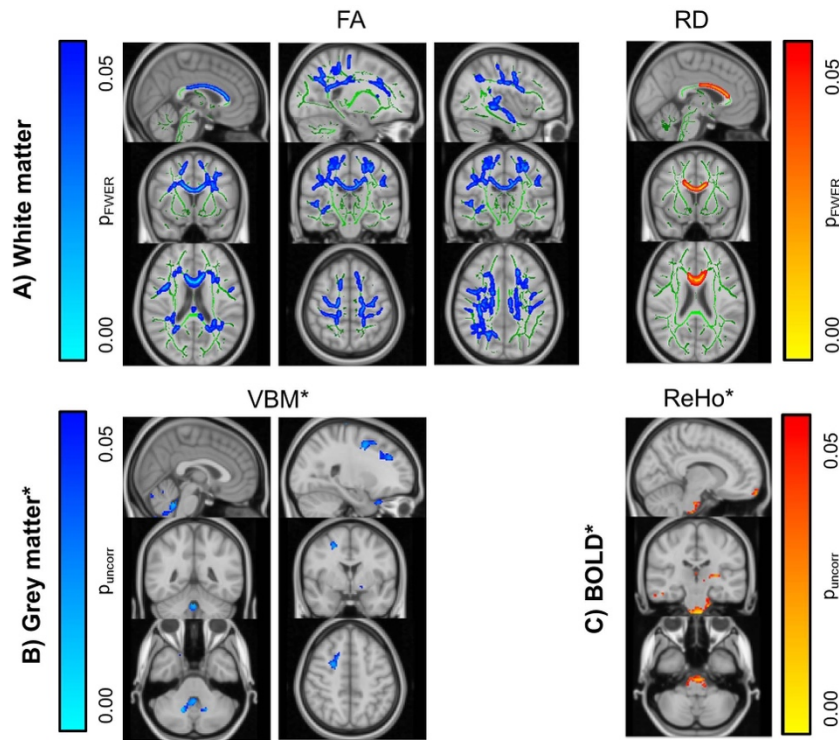


Figure 5.3 Data-driven whole-brain analyses. The anatomical associations of pseudobulbar affect (ELQ) with white matter metrics (TBSS), grey matter indices (VBM and CTh analyses) and resting state functional MRI (BOLD - ReHo) to PBA (using ELQ scores) over the entire cerebrum were investigated. TBSS identified significant results after TFCE-correction for FA and RD (A), including significant correlations between more severe PBA and widespread FA reductions (CC, CST and association tracts) and RD increases in the CC. No voxels survived TFCE-correction for GM and BOLD analyses. Given the exploratory nature of the study, uncorrected p-maps thresholded at $p_{\text{uncorr}} \leq 0.05$ are also presented for VBM (B) and ReHo (C) revealing GM density reductions in the cerebellum and frontal cortical regions in association with higher ELQ (B) as well as increased uncorrected ReHo in the brainstem (C). CTh revealed on significant associations on corrected or uncorrected statistical maps.

* denotes FWE-uncorrected statistical maps, thresholded at $p_{\text{uncorr}} \leq 0.05$; otherwise maps are TFCE-corrected; cool colours indicate inverse correlations, hot colours denote direct correlations. Voxel locations are provided in MNI152 2mm standard space along with their original label names as per the relevant atlases provided in brackets: Upper row, left to right (labels according to JHU ICBM-DTI-81 WM labels atlas / XTRACT HCP Probabilistic Tract Atlases): 1) 91-138-93: Body of CC; 2) 67-107-127: right CST – also showing left CST, e.g. at 116-107-126; 3) 50-107-107: not labeled, suggestive of Corticobulbar Tract – also widespread parts of Arcuate / Superior Longitudinal / Middle Longitudinal / Inferior Longitudinal Fascicles; 4) 91- 138-93: Body of Body of CC
Lower row, left to right (labels according to Harvard-Oxford Cortical / Subcortical Atlases): 1) 44-39-18: Vermis; 2) 33-63-60: Precentral Gyrus – also showing Middle Frontal Gyurs at 33-78-51; 3) 40-51-14: Brainstem – also showing Frontal Pole, e.g. at 40-96-27, and left Putamen (60-51-38). Abbreviations: AD = axial diffusivity, BOLD = blood-oxygen-level-dependent, CC = corpus callosum, CST = Corticospinal Tract, CTh = cortical thickness, DTI = diffusion tensor imaging, ELQ = emotional lability questionnaire, FA = fractional anisotropy, FWE(R) = family wise error (rate), GM = gray matter, ICBM = International Consortium for Brain Mapping, HCP = Human Connectome Project, JHU = Johns Hopkins University, PBA = pseudobulbar affect, RD = radial diffusivity, ReHo = regional homogeneity, TBSS = tract-based spatial statistics, TFCE = threshold-free cluster enhancement, VBM = voxel-based morphometry, WM = white matter, XTRACT = cross-species tractography

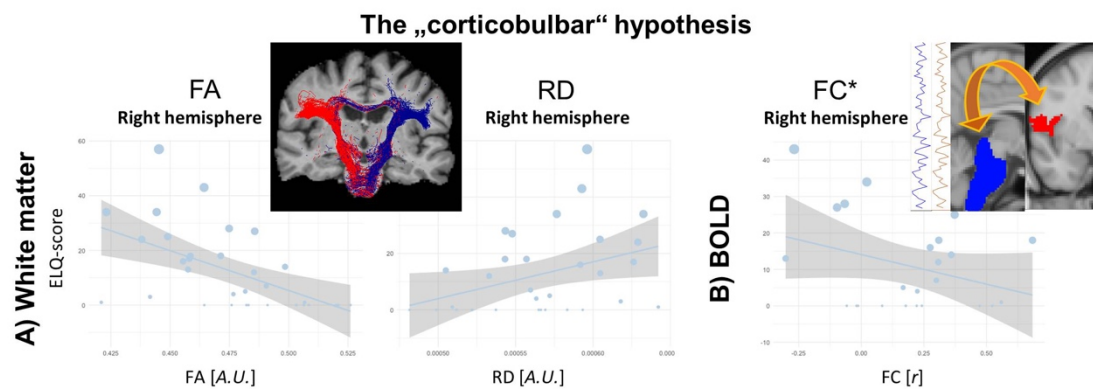


Figure 5.4 The evaluation of the “corticobulbar hypothesis”. Associations of pseudobulbar affect with white matter metrics (mean FA / RD / AD of the corticobulbar tract), grey matter indices (medullary volume, thickness of the bulbar motor cortex) and the functional connectivity between the brainstem and bulbar cortex (BOLD) were investigated. Significant associations were identified between the right-hemispheric corticobulbar tract and ELQ scores (A) including FA ($p = 0.006$) and RD ($p = 0.026$). For BOLD (B), a tendency ($p = 0.080$) for decreased FC between the brainstem and the bulbar segment of the primary motor cortex was identified. No associations with GM metrics reached significance at the alpha-level of $p < 0.05$. Statistical details are summarised in the main text and **Table 5.2**. * denotes tendency for significant association ($p < 0.10$). Abbreviations: AD = axial diffusivity, BOLD = blood-oxygen-level-dependent, CTh = cortical thickness, ELQ = emotional lability questionnaire, FA = fractional anisotropy, FC = functional connectivity, FWE(R) = family wise error (rate), GM = gray matter, PBA = pseudobulbar affect, RD = radial diffusivity, WM = white matter

tract showed particular associations with ELQ scores, as measured by FA reductions ($p = 0.006$) and RD increases ($p = 0.026$, **Figure 5.4A**). For the left hemispheric corticobulbar tract, we only identified statistical trends for the analogous associations (FA: $p = 0.065$, RD: $p = 0.074$). Similar to the above correlations with structural corticobulbar connectivity metrics, functional measures also revealed associations. Lower functional connectivity (FC) between the bulbar segment of the right motor cortex and the medulla was associated with a trend of higher ELQ scores ($p = 0.080$, **Figure 5.4B**). No associations were detected based on medullary volumes, cortical thickness of the bulbar segment of the motor cortex or axial diffusivity of the corticobulbar tracts.

The Cerebellar hypothesis: No supporting radiological findings identified

In our final analysis stream, we have evaluated a panel of structural and functional cerebellar metrics, but no associations were identified with pseudobulbar symptoms. Statistical details

are provided in **Table 5.3** Cerebellar grey matter volume and thickness did not show associations with ELQ scores. Similarly, voxelwise cerebellar GM correlations did reach significance either on VBM or for CTh following FWE-corrections. However, the inspection of the FWE-uncorrected p -maps for VBM and CTh revealed T1w signal reduction (VBM) and CTh reduction (CERES) of the vermis in association with increased ELQ. Similarly, none of the structural WM connectivity metrics (FA, RD, AD) of the cerebellar peduncles (Lt/Rt SCP, MCP, Lt/Rt ICP) yielded statistically relevant associations with PBA. Only a trend of association was identified between higher ELQ scores and higher AD in the left SCP ($p = 0.0501$). In terms of functional connectivity, neither left- nor right-hemispheric FC decreases were identified between the brainstem and the cerebellum in association with PBA on BOLD analyses.

Table 5.2 Statistics of the “corticobulbar hypothesis”, i.e. associations between corticobulbar metrics and PBA

Neuroimaging metric	General linear model, testing main effect “neuroimaging metric”					
	Left hemisphere			Right hemisphere		
	Estimate	t-value	p-value	Estimate	t-value	p-value
Gray matter						
Medulla volume	-8.92e-7	-0.221	0.827	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Bulbar CTh	-4.25e-3	-1.036	0.310	-0.002	-0.593	0.558
White matter						
FA corticobulbar tract	-6.77e-4	-1.926	0.065+	-9.75e-4	-3.010	0.006*
RD corticobulbar tract	1.04e-6	1.861	0.074+	1.25e-6	2.359	0.026*
AD corticobulbar tract	3.25e-7	0.690	0.497	1.12e-7	0.248	0.806
BOLD						
FC brainstem-bulbar cortex	-6.22e-4	-0.165	0.871	-0.007	-1.853	0.080+

All statistics were derived from a GLM, investigating the main effect of the respective neuroimaging metrics (modeled as independent variable) on the dependent variable (ELQ scores). Potential confounding effects of age, sex, handedness and years of education were accounted for. One-sided testing scheme was used for all analyses, specifically testing the association between more severe PBA and decreased radiological integrity metrics i.e. decreased volume / CTh / FA / FC, increased RD / AD. As the medulla is a single structure, its volumetric analyses are only presented under the “left hemisphere” column. Bold font denotes statistical significance at an alpha level of $p \leq 0.05$; + denotes tendency for statistical significance at $p \leq 0.10$. Abbreviations: AD = axial diffusivity, BOLD = blood-oxygen-level-dependent, CTh = cortical thickness, ELQ = emotional lability questionnaire, FA = fractional anisotropy, FC = functional connectivity, GLM = general linear model, PBA = pseudobulbar affect, RD = radial diffusivity

Table 5.3 Statistics of the “cerebellar hypothesis”, i.e. associations between cerebellar metrics and PBA

Neuroimaging metric	General linear model, testing main effect “neuroimaging metric”					
	Left hemisphere			Right hemisphere		
	Estimate	t-value	p-value	Estimate	t-value	p-value
Gray matter						
Cerebellar CVol	-3.28e-5	-0.673	0.507	-1.73e-5	-0.379	0.707
Cerebellar CTh	0.004	0.787	0.441	0.002	0.458	0.652
White matter						
Superior Cerebellar Peduncle (SCP)						
FA SCP	-2.71e-4	-0.972	0.340	-3.47e-4	-1.354	0.188
RD SCP	-1.80e-7	-0.369	0.715	2.56e-7	0.493	0.626
AD SCP	-1.37e-6	-2.059	0.050+	-7.45e-7	-1.032	0.312
Middle Cerebellar Peduncle (MCP)						
FA MCP	-3.63e-4	-1.106	0.279	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
RD MCP	-9.43e-9	-0.015	0.988	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
AD MCP	-9.76e-7	-1.292	0.208	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
Inferior Cerebellar Peduncle (ICP)						
FA ICP	-4.43e-4	-1.021	0.317	-2.26e-4	-0.615	0.544
RD ICP	1.28e-7	0.157	0.876	3.84e-7	0.530	0.601
AD ICP	-9.00e-7	-0.862	0.396	5.84e-8	0.063	0.950
BOLD						
FC BS-cereb	0.002	0.309	0.761	-0.001	-0.242	0.812

All statistics were derived from a GLM, investigating the main effect of the respective neuroimaging metrics (modeled as independent variable) on the dependent variable (ELQ scores). Potential confounding effects of age, gender, handedness and years of education were accounted for. One-sided testing scheme was used for all analyses, specifically testing the association between more severe PBA and decreased radiological integrity metrics i.e. decreased volume / CTh / FA / FC, increased RD / AD. As the MCP was evaluated as a single structure, its analyses are only presented under the “left hemisphere” column. Bold “+” denotes tendency for statistical significance at $p \leq 0.10$. Abbreviations: AD = axial diffusivity, BOLD = blood-oxygen-level-dependent, BS = brainstem cereb = cerebellum, CTh = cortical thickness, CVol = cortical volume, ELQ = emotional lability questionnaire, FA = fractional anisotropy, FC = functional connectivity, GLM = general linear model, MCP = Middle Cerebellar Peduncle, ICP = Inferior Cerebellar Peduncle, PBA = pseudobulbar affect, RD = radial diffusivity, SCP = Superior Cerebellar Peduncle, WM = white matter

5.4 Discussion

In accordance with the classical pathophysiological model of PBA, our findings implicate the structural and functional disconnection of the bulbar segment of the motor cortex and the brainstem. Our initial data-driven, whole-brain analyses revealed voxelwise correlations along the descending corticospinal/corticobulbar tracts. Uncorrected morphometric analyses also revealed a trend for association with cerebellar grey mater metrics. In line with the two most popular pathophysiological models, cortico-bulbar and cerebellar hypotheses were specifically evaluated in post hoc region-of-interest analyses.

The integration of research findings in PBA is hindered by differences in terminology, diagnostic criteria and screening practices in different geographical regions and in different neurological conditions. Despite the definition of specific thresholds on various PBA rating scales, the presence of PBA is unlikely to be binary (present or not) but likely to be along a spectrum of symptom severity which may be progressive in certain conditions (200). While the 1969 *Poeck's* criteria (201) centres primarily on context-inappropriate emotional manifestations, more recent criteria are more permissive, recognising presentations across the whole spectrum of PBA (160, 202). The Revised “involuntary emotional expression disorder” (IEED) criteria (160) encompass episodes that are merely disproportionate to the emotive stimulus and are different from the patient’s emotional reactivity prior to disease onset. Supportive clinical features, such as outbursts of anger, smiling, yawning are increasingly considered (162, 202-204). Given the spectrum of manifestations we did not stratify patients into symptomatic or asymptomatic individuals based on pre-defined thresholds on the ELQ scales, but used their absolute ELQ score values in correlation models. Our clinical findings (**Table 5.1**) also underscore that the degree of pseudobulbar affect is independent from anxiety, depression and physical disability and not a direct correlate of the above disease dimensions. This is in line with the conceptualisation of PBA as mood incongruent episodes of emotional manifestations and also consistent with studies in ALS where typically PBA cannot be linked directly to underlying depression (57, 60, 161, 205, 206). The most commonly used screening scales include the 1993 Pathological laughing and crying scale (PLACS) (207), the self-reported Center for Neurologic Study-Lability Scale (CNS-LS)(208, 209) which has been extensively utilised in recent pharmacological trials (210-213). The “emotional lability questionnaire” (ELQ) has also been consistently utilised in MND. It takes symptoms into to up to four weeks prior to screening into account, which helps to ascertain patients with experience frequent symptoms (214, 215). ELQ also evaluates the caregiver’s

views on the patients PBA, but in MND, the patient and caregiver scores are typically concordant (214). While a multitude of validated instruments exist to appraise the severity of PBA, the specific quality of life (QoL) ramifications of PBA are relatively understudied (162, 205), despite evidence that it not only impacts on the social life of affected patients (216) but also on caregivers (217). Even though multiple therapeutic approaches exist, these are not always effective and divergent strategies are typically used in different conditions. In neurovascular conditions, selective serotonin reuptake inhibitors (SSRIs) are typically trialled first (204), but tricyclic antidepressants (TCAs) are also widely utilised (207). In MND, amitriptyline (218) and duloxetine (219) are often used as first-line therapies. Memantine is sometimes used in Alzheimer's disease. Since 2010, dextromethorphan / quinidine (Dx/Q) is increasingly prescribed and randomised, double-blinded studies have confirmed its efficiency in ALS (212). Despite the multitude of therapeutic options, achieving full symptom control can be challenging and often a combination of drugs are prescribed.

In our study we have exclusively included patients with primary lateral sclerosis. PLS is a low-incidence neurodegenerative condition with one of the highest rates of pseudobulbar affect in any neurological condition, making it an ideal cohort to study to likely underpinnings of this clinical syndrome. Radiologically, PLS is classically associated with motor cortex, corticospinal tract and corpus callosum degeneration (68-71), but extra-pyramidal involvement is also increasingly recognised (63, 135). While there are considerable radiological similarities between ALS and PLS (220), and PBA is also prevalent in ALS, PLS is associated with a considerably better prognosis than ALS. It has been previously stipulated that frontotemporal pathology may play a role in the aetiology of PBA, but as demonstrated in our cohort, there are no direct associations noted on any of the whole- brain, data-driven analyses between frontotemporal integrity and ELQ scores. Our voxelwise and vertex wise, grey- and white matter analyses did not capture significant associations between frontotemporal metrics and PBA.

The strong association noted between PBA and corticobulbar tract integrity may have broader clinical and diagnostic ramifications in motor neuron diseases and potentially in other neurological syndromes. Compared to the plethora of routinely evaluated pathological upper motor neuron (UMN) signs in the limbs; Babinski, Oppenheim, Gordon, Chaddock, Hoffmann's, Tromner's etc. there is a paucity or reliable bulbar UMN signs. The jaw- jerk

(masseter reflex) is often equivocal early in neurodegenerative conditions, when it may be difficult to elicit and reliably interpret. Similarly spastic dysarthria may be difficult to confidently ascertain unless reviewed by expert speech pathologist or trained speech and language therapists (1). Accordingly, in the correct context, PBA may be an excellent clinical indicator of corticobulbar tract involvement or cortico-medullary disconnection. This in turn may have diagnostic implications, particularly in ALS, where concomitant LMN signs may be readily identified on clinical or neurophysiological assessment (1). As the quantitative analyses of corticobulbar tract integrity based on DWI, fMRI, or transcranial magnetic stimulation (TMS) are time-consuming, expensive and many MND patients do not tolerate MRI scanning (221), the presence of marked PBA may be interpreted as evidence of corticobulbar tract pathology.

Our whole-brain and targeted cerebellar analyses did not reach significance, although a trend of association was identified between higher ELQ scores and higher AD in the left SCP ($p = 0.0501$). Notwithstanding our findings in PLS, cerebellar involvement has been previously linked to PBA in ALS (59, 61) and relatively selective cerebellar signatures have been consistently described in various motor neuron disease phenotypes (7, 114, 115, 222). While frank ataxia is seldom observed in PLS or ALS, subclinical cerebellar involvement is thought to contribute to neuropsychological deficits, exacerbate dysarthria, worsen gait impairment, and potentially impact on dysphagia or respiratory patterns (115, 117, 118, 123, 124).

From a methodological perspective, the multimodal approach adopted in our study permits the appraisal of specific measures. There is a relatively strong concordance between structural and function imaging metrics in highlighting structural and functional disconnection between brain regions. Structural connectivity metrics however captured statistically significant association both in whole-brain and ROI analyses. We have investigated there diffusivity metrics, FA, AD and RD, but only FA and RD alterations were associated with clinical measures in corticobulbar tracts. In the cerebellar analysis stream however, a trend of association was identified between higher ELQ scores and higher AD in the left SCP. These observations confirm the merits of a multimodal imaging protocol including a panel of structural and functional measures and more importantly, the systematic assessment of multiple diffusivity indices. Cortical thickness is one of the most commonly assessed radiological metric in MND, in no small part, due to the excellent semi-automated pipelines. Interestingly, in our study measures of PBA did not reveal associations with focal grey matter atrophy neither on the

cerebral vertex or in the cerebellum. This confirms that the radiological correlates of specific symptoms may not be solely driven by unifocal anatomical changes, rather results from the dysfunction of complex neuronal networks often with multiple cortical, subcortical and white matter components (30, 39, 83, 108, 122). Connectivity analyses should therefore ideally be incorporated in exploratory studies aiming to decipher the underpinnings of specific symptoms. We also note the relative symmetry of our findings; imaging metrics of bilateral structures were evaluated in the left and right hemispheres separately and resulted in concordant findings. We also note that none of the patients carried the *C9orf72* HRE which may have introduced a potential confound given the unique clinical and radiological correlates of that genotype (76, 151, 223).

This study is not without limitations. While comprehensive genetic and clinical assessments were performed, we have only evaluated a relatively small cohort of patients. A single PBA battery was administered, and a cross-sectional study design implemented. Given the evidence of progressive radiological changes in PLS (55, 224), this approach precludes the characterisation of worsening PBA over time. While there may be a benefit of assessing a diagnostically homogenous cohort, the inclusion of a second condition where PBA is prevalent would have been desirable. Our findings may be specific to PLS and alternative pathomechanisms may contribute to PBA in other conditions. Notwithstanding these limitations, our imaging study confirms the contribution of cortico-medullary disconnection to pseudobulbar affect.

5.5 Conclusions

Pseudobulbar affect in PLS is associated with structural and functional cortico-medullary disconnection. Additional cerebellar components may also play a role and it is likely that the aetiology of PBA is multifactorial with the involvement of several cerebral networks.

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7 Appendix

Table S 7.1 Associations between neuroimaging metrics and clinical scores in PLS patients.

<u>MRI-clinical associations (bulbar PUMS)</u>												
	Right hemisphere			Left hemisphere			Right hemisphere			Left hemisphere		
	esti- mate	t- value	p- value	esti- mate	t- value	p- value	esti- mate	t- value	p- value	esti- mate	t- value	p- value
AD	9.85 e3	1.45	0.157	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>						
FA	-22.1	-2.78	0.009 *	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>						
RD	1.37 e4	2.58	0.015 *	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>						
FC	3.64	2.24	0.035 *	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>						
<u>MRI-clinical associations (UL PUMS)</u>							<u>MRI-clinical associations (LL PUMS)</u>					
CT	- 0.03 9	- 0.050	0.961	0.112	0.121	0.904	0.647	0.900	0.374	0.182	0.269	0.790
AD	1.65 e4	2.67	0.012 *	1.94e 4	3.05	0.005	1.62e 4	1.93	0.063 +	6.91e 3	1.21	0.235
FA	-30.5	-2.60	0.014 *	-13.0	-1.11	0.276	-12.1	-1.67	0.104	-13.0	-1.57	0.126
RD	2.2e 4	3.37	0.002 *	1.51e 4	2.26	0.031	1.11e 4	2.05	0.049 *	9.51e 3	1.77	0.087 +
FC	-1.89	- 0.822	0.420	-3.26	-1.56	0.133	- 0.910	- 0.453	0.655	-2.40	-1.73	0.100 +
<u>MRI-clinical associations (UL TAP rates)</u>							<u>MRI-clinical associations (LL TAP rates)</u>					
CT	-5.60	- 0.507	0.616	-4.60	- 0.357	0.723	-12.4	-1.66	0.107 +	-6.27	-1.02	0.316
AD	- 1.43 e5	-1.53	0.136	- 2.12e 5	-2.27	0.031 *	- 1.42e 5	-1.41	0.171	- 7.44e 4	-1.31	0.201
FA	4.34 e2	2.62	0.014 *	2.64e 2	1.57	0.126	1.62e 2	2.21	0.036 *	1.16e 2	1.39	0.175
RD	- 2.5e 5	-2.65	0.013 *	- 2.3e5	-2.45	0.021 *	- 1.27e 5	-2.24	0.033 *	- 8.8e4	-1.64	0.113
FC	-16.9	- 0.644	0.527	32.6	1.42	0.171	20.4	1.33	0.200	21.8	1.68	0.109 +

Associations between (lateralized) clinical scores and matched hemispheric neuroimaging metrics are provided within this table, i.e. right-hemispheric neuroimaging metrics were

correlated to left-lateralized PUMS scores, etc. Notice that for the bulbar analyses, we could not differentiate between left and right-hemispheric PUMS scores. We therefore provide association analyses between the bulbar PUMS scores and the averaged neuroimaging metrics (across hemispheres) in the columns “right hemisphere”. All statistics were derived from a (cross-sectional) linear model, correcting for age, gender and handedness, using the final available MRI data for each PLS patient.

Abbreviations: AD = axial diffusivity, CT = cortical thickness, FA = fractional anisotropy, FC = functional connectivity, LL = lower limb, MRI = magnetic resonance imaging, *n.a.* = not applicable, PLS = primary lateral sclerosis, PUMS = Penn Upper Motor Neuron Score, RD = radial diffusivity, TAP = tapping rate, UL = upper limb), + = approaching significance at an alpha-level of $p \leq 0.10$ (uncorrected), * = significant at an alpha-level of $p \leq 0.05$ (uncorrected)