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ORIGINAL COMMUNICATION

Loss of brainstem white matter predicts onset and motor neuron symptoms in *C9orf72* expansion carriers: a GENFI study

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Abstract

Background and objectives The *C9orf72* expansion is the most common genetic cause of frontotemporal dementia (FTD) and/or motor neuron disease (MND). Corticospinal degeneration has been described in post-mortem neuropathological studies in these patients, especially in those with MND. We used MRI to analyze white matter (WM) volumes in presymptomatic and symptomatic *C9orf72* expansion carriers and investigated whether its measure may be helpful in predicting the onset of symptoms.

Methods We studied 102 presymptomatic *C9orf72* mutation carriers, 52 symptomatic carriers: 42 suffering from FTD and 11 from MND, and 75 non-carriers from the Genetic Frontotemporal dementia Initiative (GENFI). All subjects underwent T1-MRI acquisition. We used FreeSurfer to estimate the volume proportion of WM in the brainstem regions (midbrain, pons, and medulla oblongata). We calculated group differences with ANOVA tests and performed linear and non-linear regressions to assess group-by-age interactions.

Results A reduced WM ratio was found in all brainstem subregions in symptomatic carriers compared to both noncarriers and pre-symptomatic carriers. Within symptomatic carriers, MND patients presented a lower ratio in pons and medulla oblongata compared with FTD patients. No differences were found between presymptomatic carriers and non-carriers. Clinical severity was negatively associated with the WM ratio. *C9orf72* carriers presented greater age-related WM loss than non-carriers, with MND patients showing significantly more atrophy in pons and medulla oblongata.

Discussion We find consistent brainstem WM loss in *C9orf72* symptomatic carriers with differences related to the clinical phenotype supporting the use of brainstem measures as neuroimaging biomarkers for disease tracking.

Keywords Frontotemporal dementia · *C9orf72* · GENFI · Brainstem

Introduction

Frontotemporal dementia (FTD) refers to a heterogeneous group of neurodegenerative disorders that mainly affects the frontal and temporal lobes of the brain producing behavioral and language impairment [1]. Amyotrophic lateral sclerosis (ALS) is the most frequent motor neuron disease. It is caused by the neurodegeneration of motor neurons and the corticospinal and corticobulbar tracts leading to progressive weakness and muscular atrophy [2]. Due to the scientific advances in the last decades, it is now recognized that

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FTD and ALS are part of a clinical, neuropathological, and genetic continuum [3–6].

Although frequency varies geographically, the pathological hexanucleotide expansion in the *chromosome 9 open reading frame 72* (*C9orf72)* gene is the most common genetic cause of FTD, and ALS [7, 8]. The *C9orf72* repeat expansion is inherited with an autosomal dominant pattern with almost full penetrance leading to disease onset at a mean age of 58 years, although a wide range of age of onset (20–90 s) has been described [9]. The correlation between parental age at onset and individual age at onset for *C9orf72* expansion carriers is weak ($r=0.32$), and thus, not useful for individual predictions [9]. In the same way, whether the symptom onset would appear in form of FTD, or ALS remains unpredictable. However, future disease-modifying drugs might be useful for both clinical phenotypes and treatments might be more useful when used in early or even presymptomatic phases of the disease. For that reason, there is a need for biomarkers that are able to provide information about the proximity of onset and track disease progression in both phenotypes. In this sense, cohorts of mutation carriers, such as the genetic frontotemporal initiative (GENFI), provide the opportunity to study the first stages of the disease and to identify markers of symptom onset and progression [10].

Previous studies have described structural changes in presymptomatic FTD subjects using brain MRI [11–15]. Concerning *C9orf72* carriers, previous studies have shown presymptomatic brain changes in the thalamus, cerebellum, hippocampus, amygdala, and hypothalamus [16, 17]. Most of these studies have focused on grey matter. In contrast, white matter (WM) degeneration has received comparatively less attention but demonstrates early and widespread WM integrity loss in *C9orf72* carriers [18].

The neuropathological examination of ALS patients reveals loss of motor neurons and the consequent degeneration of the corticospinal and corticobulbar tracts [19, 20]. This degeneration leads to lateral sclerosis of the spinal cord which gives the name to the disease. In addition to spinal cord changes, ALS patients also present relevant atrophy of the white matter areas that contain the corticospinal and corticobulbar tracts at the brainstem, especially the pyramids in the medulla oblongata. Previous work has demonstrated that changes in the spinal cord and brainstem in ALS can be detected in vivo using structural MRI [21, 22]. In a recent study, Querin et al. reported significant WM reduction in the spinal cord of presymptomatic *C9orf72* carriers using cervical cord MRI [23]. Assessing WM changes in the brainstem presents some potential benefits from cervical spinal cord evaluation, as the possibility of being measured with other brain changes in the brain MRI.

In this work, we investigate the utility of brainstem WM loss as a biomarker for *C9orf72* patients. We hypothesize that symptomatic *C9orf72* carriers would present more WM loss in the brainstem compared to non-carriers, especially in those patients with motor neuron symptoms. We also aim to study whether WM loss is identifiable in presymptomatic *C9orf72* carriers.

Materials and methods

Participants

Two hundred thirty five participants' data were obtained from the data freeze 4 (DF4) of the GENFI, an international multicenter study of known carriers of a pathogenic mutation or at risk of carrying a mutation because a first-degree relative was a known symptomatic carrier [11]. Symptomatic subjects were FTD or ALS patients carrying the *C9orf72* pathogenic expansion. Presymptomatic and noncarriers subjects were all first-degree relatives of *C9orf72* mutation carriers who consent to be tested for their genetic status.

All participants' imaging data were acquired at each time point using 3 T on scanners from three different manufacturers: Philips Healthcare (Koninklijke Philips NV, Amsterdam, Netherlands), GE Healthcare Life Sciences (General Electric, Boston, MA, USA) and Siemens Healthcare Diagnostics (Siemens, Erlangen, Germany). Protocols were designed to harmonize across scanners and sites as much as possible [11]. Subjects were classified into four groups according to their genetic status (carriers or non-carriers) and their clinical diagnosis as follows: (a) non-carriers; (b) presymptomatic *C9orf72* carriers if no diagnostic criteria were fulfilled, (c) symptomatic *C9orf72* carriers with FTD presentations in the form of behavioral variant FTD [24] or primary progressive aphasia [25] and (d) symptomatic *C9orf72* carriers with MND presentation in form of ALS or ALS-FTD [26, 27]. The disease stage of all participants was scored following the global and sum of boxes Clinical Dementia Rating adapted to FTD patients (CDR®+NACC-FTLD) rating scale [28]. The severity of motor neuron symptoms was scored with the ALS Functional Rating Scale-Revised (ALSFRS-R), a validated rating instrument for monitoring the progression of disability in ALS patients [29]. The ALSFRS-R obtains a final index of disability by scoring 12 different motor and respiratory items from 4 (no disability) to 0 (marked disability). Written informed consent was obtained from all participants. All procedures were approved by local ethics committees at each site.

MRI acquisition and processing

Participants underwent a 1.1-mm isotropic resolution volumetric T1 MRI imaging on a 3T scan using the sequences defined within the GENFI consortium. Nineteen scanners were used across different sites. MRIs of all subjects were downloaded from GENFI database and processed using

FreeSurfer version 6.0 (http://surfer.nmr.mgh.harvard.edu/) in the same center.

After the standard FreeSurfer segmentation and parcellation [30–32], we used an additional FreeSurfer pipeline to segment the brainstem region and its three main structures (midbrain, pons, and medulla oblongata) [33]. Figure 1 represents the imaging methodology to obtain the brainstem region segmentation. We assessed the WM parcel for the brainstem structures by multiplying each of the regions by the WM mask. To remove the effect of brain size, we calculated the ratio of WM for each of its structures (midbrain, pons, and medulla oblongata) using the total volume of the corresponding region (region-WM volume/region-whole volume). All images were visually inspected and manually corrected when needed.

Statistical analysis

Differences in demographic data between groups were assessed using ANOVA test for continuous variables and Fisher test for dichotomous data. Post-hoc studies were assessed for both cases to identify the pair-wise group differences, using *T*-tests or Fisher test accordingly. Statistical significance was set at $p < 0.05$, with corrections for multiple comparisons using the Benjamini–Hochberg procedure.

We used ANOVA test to study group differences in the WM ratio for the brainstem subregions. Age at baseline, sex and scanner were used as covariates. Then, Tukey's HSD test was used to identify pairwise differences between groups with Benjamini–Hochberg corrections for multiple comparisons. We compared the non-carriers, the presymptomatic carriers, carriers with FTD, and carriers with MND with the same procedure. Differences in the WM ratio between CDR® + NACC-FTLD global stages were assessed using Kruskal–Wallis test for all carriers, while Spearman's rank correlation coefficient was used to study the relationship between the WM ratio and the CDR® + NACC-FTLD sum of boxes and the ALSFRS-R. We evaluated multiple linear and non-linear regressions (logarithmic, polynomial to the second, third and fourth order) to test the association between the WM ratio (dependent variable) and the genetic status, age, and their interaction. For these analyses we added scanner and sex as covariates. Models were compared using R^2 and the Akaike information criterion (AIC). R (https://www.r-project.org/) version 4.0.5 was used for all analyses.

Results

Demographic and clinical characteristics of participants

After the data quality assessment the sample was reduced to 229 participants due to the segmentation problems identified. The final sample used in the analyses included: 102 presymptomatic carriers, 52 symptomatic carriers (41 FTD and 11 ALS or ALS-FTD), and 75 non-carriers (Table 1). Some of the acquisitions ($N = 43$ subjects) had a limited Field of View, so it was not possible to measure the entire medulla oblongata ROI. Thus, these images were not included in the sub-analyses of this region (21 presymptomatic, 7 symptomatic, and 15 non-carriers).

We found significant differences between the four groups (non-carriers, presymptomatic, symptomatic-FTD, symptomatic-ALS) in sex and age. Both symptomatic groups were older than the non-carriers and presymptomatic groups ($p < 0.0001$). Therefore, these variables were included as covariates in all further analyses. No significant differences were found in any demographic or clinical variables between non-carriers and presymptomatic carriers (Table 1).

Group differences in brainstem WM ratio

Non-carriers showed WM ratios very close to 1 (0.96 for the midbrain, 0.99 for the pons, and 0.97 for the medulla). No differences were found in any region between the presymptomatic and the non-carrier groups. The *C9orf72* FTD group

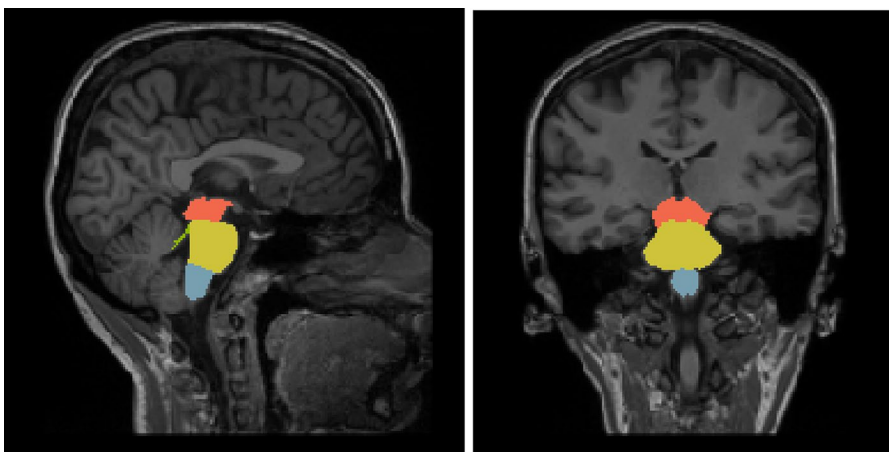

Fig. 1 The brainstem segmentation for all the matters for two different views. Orange represents the midbrain region, yellow represents the pons region and blue represents the medulla oblongata region. In this case, this subject is a healthy control

Table 1 Baseline demographics for controls, presymptomatic and both symptomatic carriers groups

	Non-carriers	*C9orf72* presymptomatic carriers	*C9orf72* FTD carriers	*C9orf72* MND carriers
Number of participants	75	102	41	11
Sex (f/m)	48/27	63/39	16/25*	4/7*
Age, years Mean (sd)	45.2 (12.6)	44.9 (11.8)	62.8 (8.4)**	62.6 (6.4)**
Age at onset, years Mean (sd)	–	–	57.2 (9.5)	59.5 (6.1)
EYO, years Mean (sd)	− 15.0 (11.6)	− 13.8 (11.9)	5.1 (6.1)**	1.4 (4.0)**
CDR®+NACC-FTLD Global Median (range)	–	–	2 (1–3)	2 (1–3)
CDR®+NACC-FTLD Sum of Boxes Median (range)	–	–	12.5 (1–22)	7.5 (1–18)

Brainstem subregions volumes and WM ratio. Show the group differences for the whole volume/WM ratio

EYO estimated years to onset, *FTD* frontotemporal dementia, *f* female, *m* male, *MND* motor neuron disease, *sd* standard deviation

*Statistical differences ($p < 0.05$) compared with non-carriers and presymptomatic carriers

**Statistical differences ($p < 0.0001$) compared with non-carriers and presymptomatic carriers

showed a lower WM ratio than non-carriers and presymptomatic carriers in all regions ($p < 0.01$ in the medulla, and $p < 0.0001$ in the midbrain and pons). The *C9orf72* MND group showed a lower WM ratio than the non-carriers and the presymptomatic carriers in all regions ($p < 0.0001$ in all comparisons). The MND group also showed a lower WM ratio than the FTD group in the medulla ($p < 0.0001$), and pons ($p < 0.0001$; (Fig. 2).

WM ratio across the severity of cognitive and motor symptoms

When studying the relationship between the WM ratio with the global CDR®+NACC-FTLD rating scale for all carriers subjects, we observed that higher clinical scores were significatively associated with lower WM ratios in all brainstem regions (Kruskal–Wallis $p < 0.001$ for all regions; Fig. 3A). Pairwise comparisons between CDR®+NACC-FTLD stages were performed for consecutive stages, depicting significant differences between the CDR = 0.5 and CDR = 1

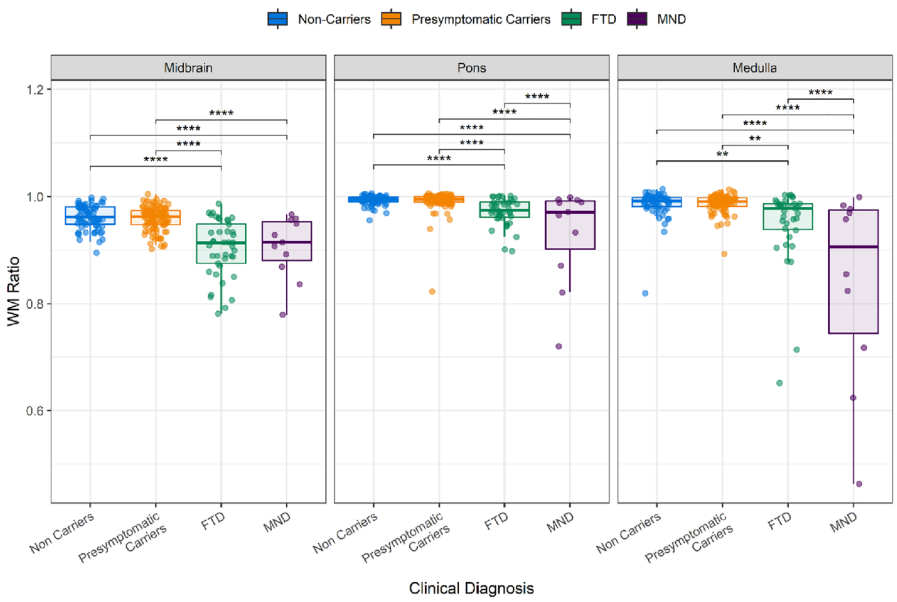

Fig. 2 Boxplot of the WM ratio volume of each brainstem region at baseline. Indicates *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, ****$p < 0.0001$

stages in the midbrain ($p < 0.05$). Additionally, moderate significant negative correlations between the WM ratio and the CDR® + NACC FTLD sum of boxes were also found for all brainstem regions (midbrain $r = -0.57$, pons $r = -0.49$ and medulla oblongata $r = -0.45$; $p < 0.0001$ all; Fig. 3B).

To assess if the WM ratio was correlated to the severity of the motor neuron symptoms, we evaluate its relationship with the ALSFRS-R score in *C9or72* carriers (Fig. 3C). We found a weak negative correlation in pons ($r = -0.37$, $p < 0.05$), but a moderate negative correlation in midbrain ($r = -0.45$; $p < 0.001$) and medulla ($r = -0.46$, $p < 0.01$).

Brainstem WM ratio and age trajectories according to the genetic status

When comparing the relationship between the WM ratio and age, we found that carriers and non-carriers showed similar trajectories until the 6th decade of life. After this

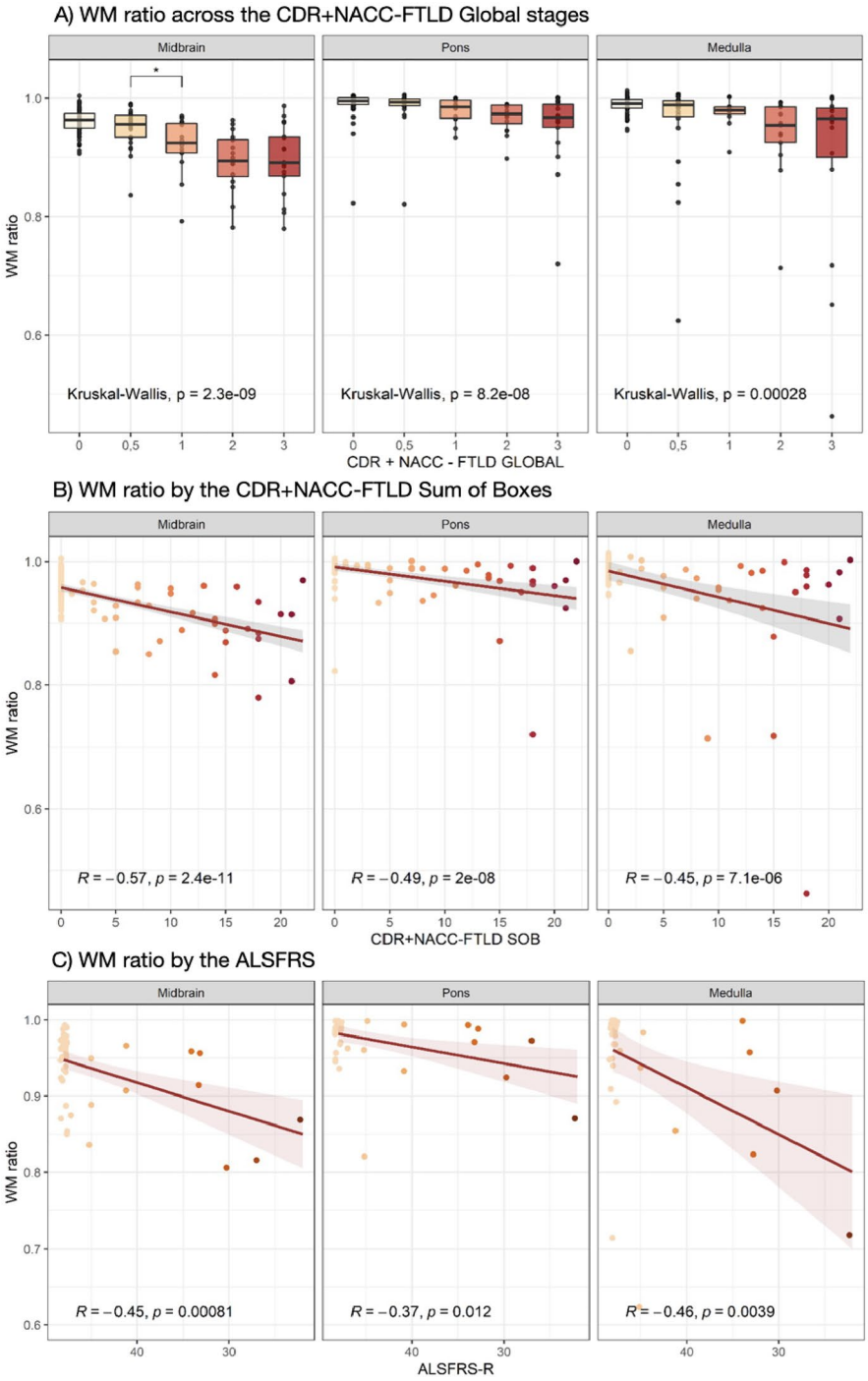

Fig. 3 **A** Boxplot of WM ratio across the *CDR® + NACC-FTLD* Global stages for the carriers' participants. Pairwise comparisons between stages were performed only for consecutive stages, finding significant differences between the 0.5 and the 1 stages in the midbrain: $*p < 0.05$, **B** Scatter plots of WM ratio by the *CDR® + NACC-FTLD* Sum Of Boxes. Red lines represent the correlation analyses, **C** Scatter plots of WM ratio by the ALSFRS-R in the different regions for the carriers' participants. Red lines represent the correlation analyses

age, carriers presented a greater loss of WM ratio than non-carriers, especially in the midbrain (Fig. 4A). The multiple linear regression comparing carriers and non-carriers showed similar results (Table 2). For both groups, age was related to lower WM ratios in the midbrain ($p < 0.001$). Carriers showed a greater loss of WM ratio by age than non-carriers in the midbrain ($p < 0.05$), suggesting a further loss of WM due to neurodegeneration. No other statistical differences were found between carriers and non-carriers. Due to the distribution of the trajectories, we also explored

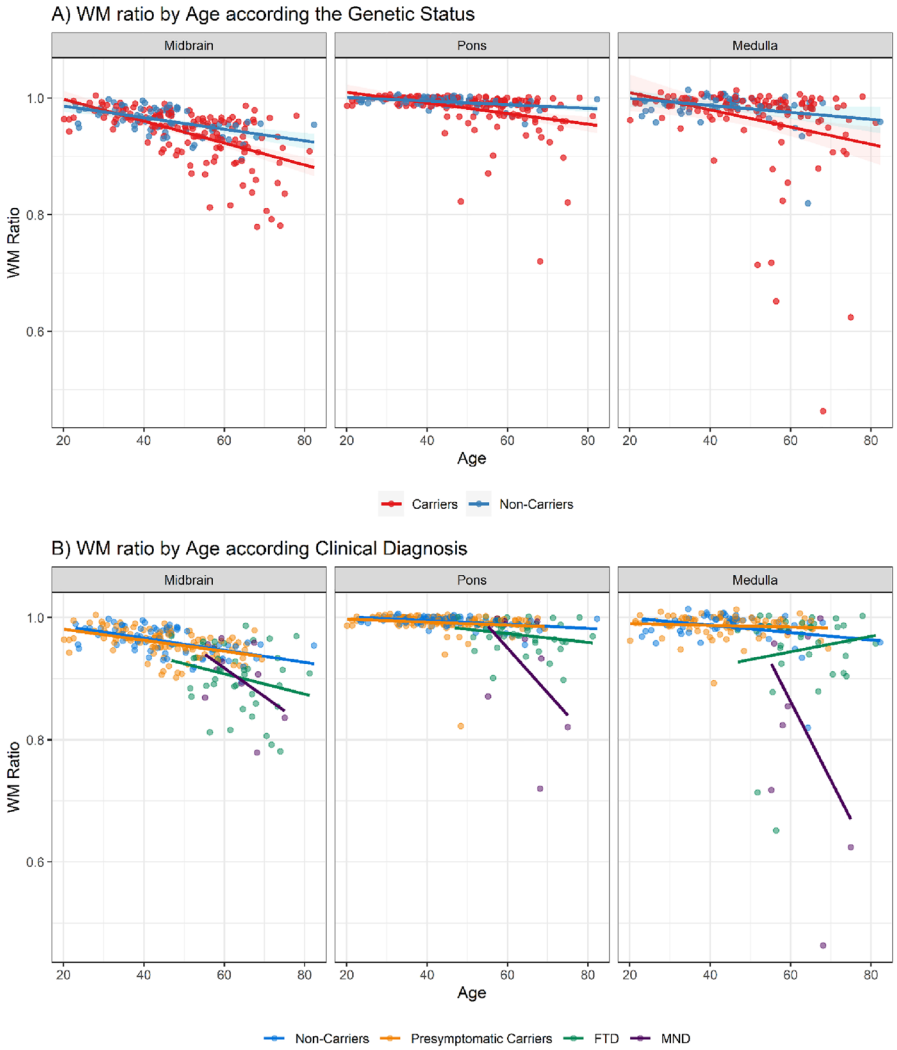

Fig. 4 Scatter plot showing the correlation between the WM ratio and age for each of the studied groups: the whole brainstem, the midbrain, the pons, and the medulla oblongata

Table 2 Multiple linear regression coefficients for comparing carriers and non-carriers

	Midbrain			Pons			Medulla		
	β	sd	*p*	*β*	sd	*p*	*β*	sd	*p*
Intercept	1.0121	0.0146	**< 0.0001**	1.0149	0.0126	**< 0.0001**	1.0284	0.0319	**< 0.0001**
Age	− 0.0010	0.0003	**< 0.001**	− 0.0003	0.0002	0.183	− 0.0007	0.0006	0.282
Scanner	− 0.0004	0.0004	0.257	− 0.0006	0.0003	0.076	− 0.0010	0.0009	0.261
Sex									
Female vs male	− 0.0056	0.0042	0.196	− 0.0050	0.0037	0.177	− 0.0159	0.0095	0.096
Genetic status									
Carriers vs noncarriers	0.02865	0.0172	0.098	0.0194	0.0148	0.192	0.0247	0.0368	0.502
Age × genetic status									
Carriers vs noncarriers	− 0.0008	0.0004	**0.019**	− 0.0005	0.0003	0.080	− 0.0007	0.0007	0.342

Significant group differences (p<0.05) are highlighted in bold

non-linear regressions, but they did not improve the linear model significatively.

Brainstem WM ratio and age trajectories according to the clinical status

Finally, we assessed the brainstem WM trajectories by age according to the clinical status to evaluate if subjects with different clinical diagnoses present different trajectories of brainstem WM during the disease. In that sense, the MND group showed a greater loss of WM by age in all regions compared to FTD patients, the medulla being the region with the highest effect of age in WM loss for this group of patients (Fig. 4B; Table 3).

Discussion

In the present study, we used brain MRI scans from the GENFI consortium to investigate whether corticospinal and corticobulbar tracts neurodegeneration is measurable in the brainstem structures of *C9orf72* carriers. Symptomatic *C9orf72* expansion carriers showed consistent alterations in brainstem WM that correlated with clinical severity. Subjects with motor neuron symptoms presented more WM loss in the brainstem than those without motor symptoms.

Brainstem neuroimaging abnormalities have been investigated by means of semi-automated volumetry methods, especially in progressive supranuclear palsy [21, 34]. Concerning *C9orf72* expansion carriers, previous work found no structural volumetric gray matter (GM) impairment in the brainstem [16, 35]. However, the evaluation of brainstem WM in *C9of72* was lacking. Here, we developed a measure of WM degeneration consisting of the proportion of the brainstem volume occupied by WM. We chose the proportion of WM instead of its whole volume to avoid differences due to different brain sizes. Assessed in the non-carriers as controls, this WM ratio showed values close to 1, reflecting that, in normal conditions, the relative GM volume in the brainstem is scarce. However, these high values might reflect an overestimation of the WM volumes. Previous neuroimaging studies have shown that small brainstem pathways might be artificially enlarged due to the inclusion of crossing fibers [36, 37]. Despite this limitation, our work found differences between groups, reflecting the utility of this measure as a neuroimaging biomarker.

We found a lower brainstem WM ratio in symptomatic *C9orf72* carriers compared to non-carriers regardless of their clinical phenotype. These differences were found in the three sub-structures (midbrain, pons, and medulla oblongata), suggesting widespread neurodegeneration of the corticospinal tracts. No differences were found between presymptomatic carriers and controls. This finding would suggest that the neurodegeneration of the WM tracts appears near the onset of the symptoms, pointing to the brainstem WM ratio as a biomarker of conversion in *C9orf72* carriers. Whether the WM neurodegeneration occurs before or after the symptom's onset remains unclear. Our study did not show WM changes in the presymptomatic carriers' group. By contrast, Querin et al. recently observed spinal cord WM atrophy in presymptomatic *C9orf72* carriers who were older than 40 years [23]. This could suggest that the spinal cord would show signs of WM alterations before the brainstem, or it could be the result of including participants who were far from the estimated year of onset in our study. The observed relationship between the brainstem WM ratio and age sheds light on this point. Overall, all subjects showed a mild loss of WM over the years with both groups, carriers, and non-carriers, showing no differences until the 6th decade of life

Table 3 Multiple linear regression coefficients for assessing the brainstem WM trajectories by age according to the clinical status

	Midbrain			Pons			Medulla		
	β	sd	*p*	*β*	sd	*p*	*β*	sd	*p*
Intercept	1.0110	0.0137	**< 0.0001**	1.0110	0.0116	**< 0.0001**	1.0146	0.0271	**< 0.0001**
Age	− 0.0010	0.0003	**< 0.001**	− 0.0003	0.0002	0.151	− 0.0006	0.0005	0.274
Scanner	− 0.0005	0.0004	0.149	− 0.0003	0.0003	0.307	− 0.0002	0.0008	0.822
Sex									
Female vs male	0.0001	0.0004	0.998	− 0.0004	0.0034	0.895	− 0.0033	0.0084	0.691
Clinical group									
Presymptomatic vs control	− 0.0083	0.0176	0.638	− 0.0056	0.0143	0.694	− 0.0175	0.0341	0.608
FTD vs control	− 0.0045	0.0380	0.905	− 0.0061	0.0308	0.841	− 0.1444	0.0730	**0.049**
MND vs control	0.1992	0.0931	**0.033**	0.3871	0.0756	**< 0.0001**	0.6220	0.1735	**< 0.001**
Age × clinical group									
Presymptomatic vs control	0.0001	0.0004	0.729	0.0001	0.0003	0.773	0.0004	0.0007	0.553
FTD vs control	− 0.0005	0.0006	0.369	0.0003	0.0005	0.500	0.0019	0.0012	0.118
MND vs control	− 0.0037	0.0015	**0.013**	− 0.0070	0.0012	**< 0.0001**	− 0.0122	0.0028	**< 0.0001**

Significant group differences (p<0.05) are highlighted in bold

when *C9orf72* carriers suffer a greater WM loss, especially in the midbrain. Of note, this decade of life coincides with the onset of symptoms reported recently by Moore et al., reinforcing the idea of the brainstem WM ratio as a possible biomarker of conversion [9].

In consonance with neuropathological studies, patients with MND showed significantly more atrophy in the pons and especially in the medulla oblongata compared to FTD. Similar results were found in the multivariate analyses, where patients presenting in form of MND suffered further loss of WM ratio than the other groups, particularly in the medulla oblongata (Fig. 4B). We hypothesize that this greater loss of WM in *C9orf72* carriers is due to the neurodegeneration of the corticospinal and corticobulbar tracts in patients who develop motor neuron symptoms. These results suggest that the brainstem WM ratio, especially in the medulla oblongata, could be an interesting biomarker to predict motor neuron symptoms in *C9orf72* carriers. This finding is particularly relevant because the form of onset in *C9orf72* carriers is highly unpredictable, and patients with motor neuron symptoms have a worse overall prognosis. Moreover, most neuroimage biomarkers studied in *C9orf72* carriers have focused on cortical atrophy, but MND patients may present only subtle cortical atrophy, especially in those with bulbar onset where, theoretically, brainstem changes were supposed to be more remarkable.

Additionally, we evaluated if the WM ratio could monitor the disease progression. For this purpose, we assessed the WM ratio across the different disease stages measured with the CDR®+NACC-FTLD scale. Here, a biological gradient was found, with patients in more advanced stages showing lower WM ratios. This loss of WM was greater in the midbrain with significant differences between the CDR = 0.5 and the CDR = 1 stages in the region. We also found a negative correlation between the CDR®+NACC-FTLD sum of boxes and the WM ratio in the brainstem. This correlation was, again, strongest in the midbrain ($r = -0.60$). We also evaluated the correlation between the WM ratio and the severity of the motor neuron symptoms in *C9orf72* carriers. A negative correlation between the WM ratio and the ALSFRS-R was found in all the brainstem regions. However, this correlation was highly influenced by subjects without motor neuron symptoms.

Our study has some limitations. First, it is important to consider that the brainstem WM visualization is challenging due to the small size of the pathways, the high density of their distributions, lower contrast, and image distortions associated with in vivo acquisitions. As mentioned before, brain volumetry could overestimate WM volumes. Despite this possible limitation, we found that our methodology is valid to find differences between groups. To support and complement our results, other MRI modalities such as Diffusion tensor imaging (DTI) may be studied in the future. Another limitation is the relatively small sample size. Even using data from a multicentric study, in some analyses, especially for the MND subgroup, the number of subjects was low, due to the low prevalence of the disease. This small number of MND patients did not allow us to study differences between subjects with bulbar or spinal onset.

In conclusion, our data suggest that WM loss in the brainstem might be a marker of clinical conversion and disease progression monitoring in C9orf72 carriers, especially in carriers presenting with motor neuron symptoms. Additional studies with extended follow-up data might be needed to confirm these findings.

Appendix

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Data availability statement The dataset analyzed for the current study is from the research consortia GENFI. Data will be shared according to the GENFI data sharing agreement, after review by the GENFI data access committee with final approval granted by the GENFI steering committee.

Declarations

Conflicts of interest JBR reports consultancy for Asceneuron, Biogen, UCB, and SV Healthcare and research grants from Janssen, Lilly, and AZ-Medimmune. JL reports speakers fees from Bayer Vital, consulting fees from Axon Neuroscience, nonfinancial support from Abbvie, compensation for part time CMO from MODAG, author fees from Thieme medical publishers and from W. Kohlhammer GbmH medical publishers, all outside the submitted work. JDR Rohrer has served as a consultant for Biogen, Ionis, Alector, Wave Life Sciences, and Astex. RSV has served in Advisory boards Meetings for Wave Life Sciences, Ionis and Novo Nordisk and received personal fees for participating in educational activities from Janssen, Roche Diagnostics and Neuroxpharma and funding to her institution for research projects from Biogen and Sage Pharmaceuticals. The other authors report no disclosures relevant to the manuscript.

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