ABSTRACT

Background and Objectives: In multiple sclerosis (MS), brain reserve serves as a protective factor against cognitive impairment. Previous research has suggested a structural counterpart in the spine—spinal cord reserve—which appeared to be associated with physical disability. This study aimed to investigate the potential of the cervical canal area (CCaA) as a proxy for spinal cord reserve in a multicentric cohort of people with MS (PwMS).

Methods: This retrospective, multicentric, longitudinal study included PwMS and healthy controls (HC) from nine European MAGNIMS sites. Baseline cervical 3D T1-weighted images were acquired, excluding poor-quality images. CCaA was estimated independently at the C2/C3 and C3/C4 levels. Expanded Disability Status Scale (EDSS) was assessed at baseline and 5-year follow-up. We analysed mean CCaA differences between groups, and the association of CCaA with baseline EDSS and disability progression using multivariable regression models adjusted for age, sex, spinal cord parenchymal fraction, and cervical cord lesions.

Results: After quality check, the cohort included 177 HC (mean age 39.8, 57.6% females), and 428 PwMS (mean age 46.5, 60.8% females), comprising 289 people with relapsing MS (PwRMS), and 139 with progressive MS (PwPMS). No significant differences in CCaA were found between HC and PwRMS at C2/C3 or C3/C4 levels. Conversely, PwPMS showed a smaller CCaA at C2/C3 level (210.51mm^2) than HC (214.62mm^2, estimated mean difference, [EMD, 95% CI] -4.11 [-6.28, -1.00], p=0.007) and PwRMS (213.68mm^2, EMD -3,17 [-5.22, -0.34], p=0.026). PwRMS also had a smaller CCaA at C3/C4 (165.16mm^2) than HC (169.67mm^2, EMD -4.51 [-5.50, -1.60], p<0.001), and PwRMS (169.44mm^2, EMD -3.81 [-5.22, -0.34], p<0.001). At C3/C4 level, CCaA and baseline EDSS were significantly associated (β−0.13, p<0.001); additionally, PwMS with clinical worsening at 5-year follow-up displayed a smaller baseline CCaA (worsened vs. stable: 167.03 mm^2 vs. 169.13mm^2, EMD -2.10 [-3.98, -023], p=0.028).

Discussion: CCaA was associated with baseline EDSS and clinical worsening in a multicentric MS cohort, suggesting the existence of spinal cord reserve. PwPMS had a smaller CCaA, indicating that reduced spinal cord reserve might be characteristic of progressive MS. Therefore, spinal cord reserve may represent a novel radiological marker for better understanding physical disability in MS.

INTRODUCTION

The concept of brain reserve, initially described in Alzheimer's disease,[1] has also been confirmed in MS.[2] It seems that the larger the brain becomes during development, the lower the risk of cognitive and physical decline becomes as caused by neurological disease. Total intracranial volume (TIV) has been used as a proxy of brain reserve,[3] representing maximal lifetime brain growth. Our recent work has explored the possible existence of a spinal cord reserve with the hypothesis that a larger spinal canal area is associated with a lower level of disability.[4] For this purpose, we measured the cervical canal area (CCaA) as a proxy of maximal lifetime spinal cord growth in the MSPATHS cohort.[5] This approach is supported by previous research showing a significant correlation between spinal cord and spinal canal areas,[6,7] with the largest values typically observed in the third and fourth decades of life,[6,8] . In later decades, the spinal canal tends to be slightly smaller. As a result, we observed that a smaller CCaA was associated with a higher disability, supporting the idea that CCaA might represent the spinal cord premorbid status. Of note, CCaA was estimated using brain magnetic resonance imaging (MRI) acquisitions, no information on disease phenotypes was available, and disability was measured by the self-reported Patient Determined Disease steps (PDDS).

In this context, we aimed to further test this hypothesis with an improved design. We estimated CCaA in a multi-centre cohort using dedicated cervical cord MRI scans in patients with different MS phenotypes, and disability was measured by the Expanded Disability Status Scale (EDSS) at baseline and 5-year follow-up. Measurements were performed using a validated semiautomated segmentation pipeline based on the Spinal Cord Toolbox[9] to assess the CCaA, using both brain and cervical cord MRIs.[10] As CCaA measurements will vary depending on the level of the cervical spinal cord under study,[8] two different spinal cord levels were explored.

Subsequently, the main objectives of the present study were: i) to compare CCaA between healthy controls (HC) and people with different MS phenotypes, assessing the CCaA at C2/C3 and C3/C4 intervertebral disc levels in dedicated cervical cord MRIs, ii) to confirm the existence of the spinal cord reserve by testing the association between the CCaA at the different spinal cord levels and disability, measured by the EDSS, at baseline, and iii) to investigate whether there is a relationship between the CCaA and disability progression at 5-year follow-up.

METHODS

Participants

This cohort study included people with MS (PwMS) who were recruited between 2010 and 2016 from nine European sites (www.magnims.eu): (1) the Amsterdam MS Centre (the Netherlands); (2) the Cemcat, Hospital Vall d'Hebron, Barcelona (Spain); (3) St. Josef Hospital Ruhr University, Bochum (Germany); (4) Queen Square Institute of Neurology, UCL, London (UK); (5) the Department of Neurology, Neurocentre of Southern Switzerland, Lugano; (6) the Department of Neurology, University of Heidelberg, Mannheim (Germany); (7) the Neuroimaging Research Unit, San Raffaele Scientific Institute, Milan (Italy); (8) the

MRI Centre "SUN-FISM," University of Campania "Luigi Vanvitelli," Naples (Italy); and (9) the Nuffield Department of Clinical Neurosciences, Oxford (UK). People with all MS phenotypes were included. HC were recruited among friends and relatives of PwMS or by local advertising. This multicentric cohort has already been used in previous studies to characterize the evolution of cervical cord atrophy,[11] and the distribution of brain grey-matter atrophy across MS phenotypes.[12]

Standard Protocols Approvals, Registrations, and Patient Consent

The project was approved by the local Ethics Committee in each Centre, and all subjects gave written informed consent before enrolment. The dataset and analyses are available from the corresponding author upon a reasonable request.

Clinical assessment and definition of disability progression

To be included, PwMS had to have stable treatment during the last six months and received no corticosteroids during the last month. Patients with a clinically isolated syndrome (CIS) suggestive of MS had to have a first episode suggestive of central nervous system demyelination and a clinical assessment within 3 months from clinical symptoms onset. Exclusion criteria for HC and pwMS were history of cervical cord/brain trauma, severe cord compression (radiologically defined) on previous MRI scans, diagnosis of MS mimickers; major comorbidities; history of drug/alcohol abuse and any other medical conditions interfering with MRI such as pregnancy.

Among MS phenotypes, there were a low number of patients with primary-progressive MS (PPMS); therefore, they were categorized into people with relapsing MS (PwRMS, including CIS and relapsing-remitting MS), and people with progressive MS (PwPMS, including secondary-progressive MS and PPMS), using present criteria for phenotype classification in all centres.[13]

Disability was measured by the EDSS score at baseline and 5-year follow-up. Confirmed clinical worsening at follow-up was defined as EDSS score increase of ≥1.5 when baseline was = 0.0, EDSS score increase of ≥1.0 when baseline EDSS was ≤5.5, or EDDS score increase of ≥0.5 when baseline EDSS was ≥6.0, as reported elsewhere.[11]

MRI acquisition

Although a strict standardization of contrast parameters was not implemented, the acquisition MRI strategy of the volumetric cord sequence was similar across sites, with the use of an isotropic (1mm x 1mm x 1mm) inversion-prepared scan,[11,14] and there were no major hardware/software updates during the study. All subjects underwent a 3D T1-weighted image (T1WI) at inclusion, covering the entire cervical cord using a 3T scanner, as well as a brain dual echo (DE) fast spin-echo or fluid-attenuated inversion recovery (FLAIR), and brain sagittal 3D T1WI.[11]

All images were visually checked by an experienced neurologist (NM). Images were excluded in presence of: cervical spondylosis with compromise of the cervical canal involving the C2-C4 segment, extreme physiological variations of the CCaA (specifically when the vertebral cavity reaches a stable diameter lower

than C2/C3 vertebral level),[8,15] and marked cervical hyperextension on acquisition. Images were also excluded due to poor MRI quality or off-center field of view.

Cervical Canal Area, spinal cord area, spinal cord parenchyma fraction, and total intracranial volume estimations

The CCaA was estimated in all participants with our in-house semiautomated segmentation pipeline based on the Spinal Cord Toolbox (Version 5.0.1),[9] as published elsewhere.[10] Briefly, segmentation of the cervical cord was performed using the *DeepSeg* algorithm.[16] Next, the posterior tips of the C2/C3 and C3/C4 intervertebral discs were manually labelled. The output from the *DeepSeg* algorithm, combined with these manual landmarks, was used to normalize the images to the PAM50 atlas.[17] Our research group previously created a cervical canal template covering from C1 to C5 in the same space as the PAM50 atlas, which was added to the predefined structures (PAM50_41, https://github.com/neuroradiologyVH/Spinal-Cord-Canal-Template/blob/main/PAM50_atlas_41.nii.gz). A spinal canal segmentation mask was also created and integrated into the atlas. The images were then normalized using the inverse normalization matrix, and the spinal canal mask was transferred to the native space. Finally, CCaA was calculated as the mean cross-sectional area over 11 slices centred on two different intervertebral disc levels: C2/C3 and C3/C4. As part of the segmentation quality control, a coefficient of variation (CV) was calculated for each CCaA measurement at the two different levels, removing subjects who displayed a CV>0.075.[4] Segmentation process failures were also removed.

The output of the pipeline also provided the mean spinal cord area (SCA), which was used to calculate the spinal cord parenchymal fraction (SCPF) as the ratio of SCA to the CCaA, and then reported as percentage. Of note, SCA and SCPF were also assessed both at C2/C3 and C3/C4 intervertebral levels.

Brain T2-hyperintense and T1-hypointense lesion volumes were quantified on DE/FLAIR scans and on 3D T1WI using the Jim software package (version 7, Xinapse Systems, Colchester, UK).[11] After refilling of T1-hypointense lesions, baseline TIV was calculated using FSL SIENAx.[11]

Statistical Analysis

The statistical analysis was conducted separately at C2/C3 and C3/C4 intervertebral disc levels. First, a descriptive analysis and a comparison between included and excluded participants were performed. The analysis included the percentage of patients in each phenotype, the mean age and disease duration, as well as the median and interquartile range (IQR) of EDSS and number of cord lesions (0,1,2,3…). Subsequently, comparisons between included HC and PwMS were performed in terms of demographic, clinical and MRI characteristics. Age- and sex-adjusted linear models were built to test for differences in CCaA between HC, PwRMS and PwPMS. To further analyse differences between MS phenotypes, we conducted a sub-analysis matching PwPMS and PwRMS by age within the C2/C3 cohort. We then assessed differences in CCaA, adjusting for sex, SCPF, and the number of cord lesions at both the C2/C3 and C3/C4 levels.

The Spearman correlation was used to investigate the relationship between CCaA and EDSS at baseline. Additionally, multivariable linear regression models adjusted for age, sex, SCPF and number of cord lesions were used to evaluate the association between EDSS and CCaA at baseline, firstly with the whole cohort,

and then by phenotypes. As the distribution of the phenotype differed between centres, no attempt was made to adjust by centre to avoid model overadjustment (see Supplementary Table). Additionally, we employed the jackknife resampling method to evaluate the robustness and stability of the association between the CCaA and baseline EDSS in the entire cohort.

We also compared the CCaA at baseline between PwMS who presented clinical worsening at 5-year follow-up to those who remained stable by using a multivariate linear regression model adjusted for age, sex, SCPF, and number of cord lesions.

Finally, we examined the relationship between TIV and CCaA using a partial correlation analysis, adjusted for age and sex, at both the C2/C3 and C3/C4 intervertebral disc levels. The analysis was firstly conducted on the entire cohort, followed by separate analyses by phenotypes.

To appraise assumptions of linear regression, we checked the normality of residuals using the Shapiro-Wilk test; homoscedasticity was evaluated with the Breusch-Pagan test, and collinearity was assessed by the variance inflation factor. The p value for significance was set at $p < 0.05$. Statistical analysis was performed with STATA 16.1 software (StataCorp).

RESULTS

An initial set of 177 HC and 428 PwMS (289 [67.5%] PwRMS, and 139 [32.5%] PwPMS) had a cervical cord 3D T1WI. After the visual quality check, 139 MRIs were removed (35 HC [19.8%], 72 [24.9%] relapsing MS and 32 [23.0%] progressive MS). Among these, 15/139 showed signs of cervical spondylosis, 5/139 had a vertebral cavity with a stable diameter lower than the C2/C3 vertebral level, 13/139 exhibited marked cervical hyperextension, 85/139 had poor MRI quality, and 21/139 had an off-centre field of view. Following quality control, the segmentation process failed in 9 subjects. Out of 457 final participants, 18 MRIs (3.9%) were removed only from the analyses of C2/C3 level, and 7 (1.5%) were removed from the analyses of C3/C4 level, since these CCaA segmentations displayed a CV>0.075 (Fig 1).

Baseline demographical, clinical and MRI data at both intervertebral levels of the final cohort can be found in Table 1. Patients with progressive MS were significantly older with a longer disease duration, a higher disability, a greater number of cervical cord lesions, and a percentage of women closer to 50%. Excluded participants had overlapping characteristics to the final cohort.

CCaA at C2/C3 intervertebral disc level

The final cohort comprised 135 HC and 304 PwMS (207 [68.1%] relapsing MS and 97 [31.9%] progressive MS). In age and sex-adjusted regression models, there were no significant differences in CCaA between HC and PwRMS (214.62mm^2 vs. 213.68mm^2, estimated mean differences [EMD, 95% CI] 0.98 [-2.87, 1.15], p=0.40), but PwPMS showed a significantly smaller CCaA (210.51mm^2) than HC (214.62mm^2, estimated mean difference, [EMD, 95% CI] -4.11 [-6.28, -1.00], p=0.007) and PwRMS (213.68mm^2, EMD -3,17 [-5.22, -0.34], p=0.026) (Fig 2). In the sub-analysis, we matched 77 PwPMS with 77 PwRMS by age. Clinical, demographic, and radiological characteristics are presented in Supplementary Table 2. Although

PwPMS exhibited a smaller CCaA compared to PwRMS, this difference did not reach statistical significance (211.09mm^2 vs. 213.34mm^2, respectively, EMD -2.24 [-5.64, 1.15], p=0.18).

In the cross-sectional analysis for the whole sample, a significant negative correlation between CCaA and EDSS was found (Spearman's *rho* -0.19, p=0.007). This association was confirmed with an age- and sex-adjusted linear model (β=−0.11; p=0.023; adjusted-R^2=0.37). However, when adjusting by SCPF and number of cord lesions, the significance disappeared (β=−0.05; p=0.26; adjusted-R^2=0.44) (Table 2). The analysis by phenotypes including all adjusting variables, showed a significant association between EDSS and CCaA in PwRMS (β=−0.19; p=0.002; adjusted-R^2=0.35), but not in PwPMS (β=0.22; p=0.051; adjusted-R^2=0.10). The application of jackknife resampling in the linear regression analysis resulted in identical coefficients of predictor variables, standard errors, and confidence intervals as those in the original model. However, the relationship between CCaA and baseline EDSS did not reach significance at this level either.

At 5-year follow-up, 85 patients (32.7%) experienced disability progression. We did not find differences in CCaA between patients with clinical worsening and those who remained stable (212.01mm^2 vs. 213.36mm^2, EMD -1.35 [-3.79, 1.10], p=0.28).

At this level, no significant partial correlation was found between TIV and CCaA, either in the entire cohort (r 0.068, p =0.18), or by phenotypes (in HC: r 0.16 p=0.11; in PwRMS: r 0.07, p=0.35, in PwPMS: r 0.05, p=0.17).

CCaA at C3/C4 intervertebral disc level

The final cohort comprised 142 HC and 308 PwMS (208 [67.5%] relapsing MS and 100 [32.5%] progressive MS). As in the C2/C3 level, there were no significant differences in CCaA when comparing HC and PwRMS (169.67 mm^2 vs. 169.44mm^2, EMD 0.23 [-1.44, 1.52], p=0.76), but again, PwPMS displayed a significant smaller CCaA (165.16mm^2) than HC (169.67mm^2, EMD -4.51 [-5.50, -1.60], p<0.001), and PwRMS (169.44mm^2, EMD -3.81 [-5.22, -0.34], p<0.001) (Fig 2). In the age-matched sub-analysis of MS phenotypes, PwPMS exhibited a significantly smaller CCaA compared to PwRMS at this level (165.17 mm^2 vs. 168.50mm^2, EMD -3.33 [-6.11, -0.55], p= 0.019)

A significant negative correlation was also found between CCaA and EDSS at baseline (Spearman's *rho* -0.34, p<0.0001). The multivariate regression model adjusted by age, sex, SCPF and number of cord lesions confirmed this association, both when including the whole cohort (β=−0.13; p=0.009; adjusted-R^2=0.43) (Table 2), and the relapsing phenotype (β=−0.16; p=0.02; adjusted-R^2=0.33). As in the C2/C3 level, the association was not significant in PwPMS (β=0.11; p=0.36; adjusted-R^2=0.01). Jackknife resampling analysis revealed that the coefficients of the predictor variables, along with the standard errors and confidence intervals, remained unchanged with and without jackknife adjustment in the whole cohort, consistent with the original model, which enhances the association between the CCaA and baseline EDSS.

At 5-year follow-up, 86 patients (32.7%) showed disability progression. Patients with clinical worsening showed a significant smaller CCaA at baseline compared to those who remained stable when adjusting by

age and sex (167.03mm^2 vs. 169.13mm^2, EMD -2.10 [-3.98, -0.23], p=0.028). However, when adjusting by SCPF and number of cord lesions, the significance disappeared (β=−0.03; p=0.60; adjusted-R^2=0.17).

At this level, there was a significant partial correlation between TIV and CCaA, adjusted for age and sex, in the entire cohort (r = 0.13, p = 0.007). This correlation was also significant in the HC group (r = 0.19, p = 0.042) and showed a trend towards significance in PwRMS (r = 0.13, p = 0.06). In contrast, no significant partial correlation was found in PwPMS.

DISCUSSION

In this work, we studied for the first time CCaA variations across people with all MS phenotypes and HC in a multicentric cohort, using our validated spinal canal segmentation tool in cervical cord MRIs.[10] Our measurements are fully in line with those documented by Kato et al.[8] We did not find differences in CCaA between HC and relapsing MS group, but progressive patients displayed a significant smaller CCaA. CCaA and baseline EDSS were associated, and smaller CCaA at C3/4, but not C2/3, was also associated with disability worsening.

In the brain reserve concept, TIV is used as a proxy for head size and maximal brain growth,[3] representing a fixed construct of brain capacity. It assumes that individuals with larger brain reserves have more neurons to lose before cognitive impairment manifests.[3] Although measuring synapse count is beyond the current capabilities of MRI, cross-sectional and longitudinal studies suggest that, at the same level of disease burden, individuals with larger TIV exhibited less cognitive decline.[2,18] Therefore, brain reserve can be conceptualized as a neuroanatomic resource reflecting structural properties of the brain that provide surplus capacity to maintain cognitive function despite substantial loss of brain material.[19]

In an attempt to translate this concept to the spinal cord and explore the possible existence of a spinal cord reserve in MS, we analysed the spinal canal area in a multicentric cohort. In the analysis across phenotypes, we observed no differences in CCaA between HC and relapsing MS group, suggesting that CCaA could serve as a surrogate measure for maximal spinal cord lifetime growth and support the testing of the spinal cord reserve concept in MS. In contrast, progressive MS patients displayed a significantly lower CCaA, both at C2/C3 and C3/C4 levels. Consequently, it seems that a smaller CCaA could be a feature of progressive forms of MS. This finding was further supported by an age-matched sub-analysis of MS phenotypes at the C3/C4 level, where PwPMS also demonstrated a smaller CCaA compared to PwRMS. Notably, there were no differences in sex distribution, and disease duration was comparable between these subgroups.

In our previous work, we showed that CCaA was independently related to self-perceived disability.[4] In that study, CCaA was estimated from brain MRI acquisitions, no information on disease phenotypes was available, and EDSS was unavailable, as disability was measured by the PDDS. The present study was undertaken to confirm the previous findings with an improved design. For this purpose, we estimated CCaA from dedicated cervical cord MRI scans at two different intervertebral disc levels. Even though a good agreement between CCaA estimations from brain and spine MRI scans has been proven, estimations from

the brain MRI acquisitions tend to be smaller and slightly less reliable.[10] Additionally, we included people with all MS phenotypes, and disability was measured by EDSS, the most widely used instrument in clinical practice and clinical trials.[20] Spearman's correlation and multivariate linear regression models, particularly at the C3/C4 level, confirmed the association between baseline EDSS and CCaA in the whole cohort. These findings support the hypothesis of a spinal cord reserve, suggesting that a larger CCaA may play a role in modulating disability in MS.

In the subgroup analysis by phenotypes, the association between CCaA and EDSS did not reach statistical significance in the progressive phenotype. This may be attributed to the narrow range of EDSS scores among PwPMS in our cohort, with 50% of these patients having EDSS scores of 6.0 or 6.5. This limited variability likely hindered the detection of significant statistical associations. Furthermore, data from PPMS and SPMS were collected from only four centres, potentially limiting the representativeness of our findings. Therefore, additional research is warranted to further explore these associations.

The jackknife resampling technique yielded nearly identical results to the original multivariate regression model using the entire cohort. Coefficients of predictor variables and the adjusted R^2 value remained unchanged. Confidence intervals generated through jackknife adjustment closely matched those from the original model. Consistent coefficients across iterations suggest high reliability, indicating minimal influence from specific data points. Overall, the jackknife method has enhanced the robustness of the association between CCaA (at C3/C4 level) and baseline EDSS.

We observed that patients with disability progression at 5-year follow-up exhibited smaller baseline CCaA at C3/C4 level in age- and sex-adjusted linear models. These results are again supportive of the concept of spinal cord reserve and point towards considering CCaA as a non-modifiable contributor for clinical progression. Admittedly, when adjusting also by SCPF and the number of cervical cord lesions, linear models did not reach significance. The potential of spinal cord atrophy[11,21,22] and the presence of cervical cord lesions[23,24] as disability predictors has been well-demonstrated. In that sense, the role of CCaA in disability worsening is likely to be modest, especially when compared to the other two mentioned, more robust, pathology-driven, variables.

Although not a primary objective of the study, we also examined the relationship between TIV and CCaA, the proxies for brain reserve and spinal cord reserve, respectively. A significant partial correlation was found at the C3/C4 level in the entire cohort and the HC group, with a trend toward significance in the relapsing MS group. However, no significant correlation was found in the progressive MS group, which we attributed to the loss of statistical power due to the smaller number of subjects in this group.

Our main hypothesis posits that the spinal canal reflects the premorbid status of spinal cord growth, and its area is related to disability in MS, with smaller CCaA associated with higher EDSS scores. In our study, PwPMS had a smaller CCaA compared to both HC and PwRMS, and this difference persisted in an age-matched subanalysis, where sex distribution and disease duration were similar. CCaA was also significantly associated with EDSS scores, supporting the spinal cord reserve hypothesis. Additionally, patients with confirmed disability progression at 5-year follow-up had smaller baseline CCaA. However, since MRI was performed at varying disease stages, initial CCaA was not reported.

The spinal canal is a flexible structure, subject to age-related changes.[25] Prior studies reported cervical spinal narrowing in healthy volunteers, with prevalence ranging from 5.3%[8] to 57.9%,[25] particularly at the C5/C6 level after the sixth decade. In PwMS, other factors like paravertebral muscle loss from deconditioning may also lead to spinal cord compression,[26,27] potentially contributing to spastic paraparesis as the disease progresses.[28,29] As PwPMS had longer disease duration and greater disability, their smaller CCaA may represent not only premorbid spinal status but also a marker of disease progression when measured during evolution. Longitudinal MRI follow-up is needed to determine whether these changes are more pronounced in progressive patients. Conversely, TIV remains stable in adulthood,[30] which could explain the observed relationship between TIV and CCaA.

In the quality check, the main reason of exclusion was poor MRI quality. Spinal cord has some particularities that make the imaging process technically challenging,[31] such as its small cross-sectional dimensions and the physiological motion with the flow of cerebrospinal fluid and respiration. Motion artifacts due to cardiac and pulmonary activity could be partially controlled with cardiac and respiratory gating.[32] There are other motion-suppression techniques to correct artifacts, applied to different sequences[33] or to the entire FOV,[34] but they usually represent a challenge. Additionally, differences in the magnetic susceptibility between bone, soft tissues and air represent a source of "noise", image distortion and loss of signal intensity, causing further field inhomogeneities and hindering the CCaA segmentation. Several post-processing approaches have been described to optimize image quality,[31] but the results are not as robust as in brain MRI. For these reasons, an accurate quality check is still crucial to obtain reliable data for subsequent statistical analysis. Additional exclusion criteria were the presence of cervical spondylosis and anatomical variations of the spinal canal, which were not as relevant as the MRI quality in this cohort.

Conversely, the number of excluded subjects based on the CV criteria was very low compared to our previous study,[4] where the CCaA segmentation was performed in brain MRIs. CCaA segmentation in dedicated cervical cord MRI has been proven to provide more stable measurements,[10] which outlines the use of spinal cord MRI in MS.

Our pipeline for estimating CCaA involves registering data to the PAM50 template,[17] followed by applying the inverse transformation to the PAM50_41 spinal canal mask,[10] rather than segmenting individual spinal canals. While using templates in brain or spinal cord MRI studies is a widely adopted approach,[17,35] it has some limitations. Since templates are based on averaged data, they may not fully capture individual anatomical differences.[36,37] Despite this, templates remain valuable for standardizing imaging and comparing datasets, especially in multicentric studies. However, direct spinal canal segmentation could be more sensitive in detecting morphological changes in PwMS and warrants further exploration. Regarding the minor discrepancies between the PAM50_41 file and the updated versions of the PAM50 template, it might be necessary to redefine the 41 label in the new template.

We obtained consistent, but not fully identical results when analysing data from C2/C3 and C3/C4 CCaA segmentations. Interestingly, results derived from the C3/C4 analysis showed stronger correlations, higher beta coefficients, and more frequent statistically significant associations in multivariate linear regression models. We hypothesized that such differences are related to the fact that the cervical canal anatomy varies along its length, showing significant decreases from C1 to C3, and achieving a more stable diameter from

C3 to C7.[8,15] Consequently, C3/C4 CCaA measurements exhibit reduced variability across the 11 slices used to calculated the spinal canal area, which is reflected by smaller SDs (see Results). Additionally, fewer participants are excluded based on the CV criteria in C3/C4 CCaA segmentations, possibly due to the more stable measurements at this level (Fig 3). All these findings support segmentations at C3/C4 level to obtain CCaA estimations. As the cervical spine is located at the periphery of the field of view in brain MRI acquisitions, gradient nonlinearity distortion effects are substantial in this area.[38] Therefore, if CCaA has to be assessed at C3/C4 level, it would be advisable to use cervical MRI acquisitions. We did not consider estimating the CCaA in lower intervertebral disc levels because; as it is reported in literature,[25,39] degenerative cervical pathology and cervical disc herniations mostly occurred in the lower segments of the cervical column, being more commonly observed at C5/C6 level. Consequently, assessing the CCaA below the C5 intervertebral level could lead to underestimations of the real spinal canal area.

Several issues should be considered in the interpretation of the findings of the present study. Firstly, MRI acquisitions were conducted in some cases over a decade ago. Replication of the present study with up-to-date MRI acquisitions to prevent the exclusion of a significant number of subjects is warranted. Secondly, the lack of follow-up MRI data prevented us from confirming whether spinal canal changes are more pronounced in the progressive phenotype. Furthermore, progressive patients were older, had longer disease duration, and exhibited a narrower range of EDSS; such biases should be avoided in future studies to better elucidate the role of CCaA in this group of patients. Finally, models were adjusted by age and sex, but not by other anthropometric parameters such as height or body mass index. Sex- and age-relate effects on spinal cord areas were well-established in different studies.[40] Conversely, normalization by height is more commonly used in studies involving spinal cord volumes,[41] where it plays a more significant role than in studies assessing areas.

CONCLUSIONS

The present study provides insights into the emerging concept of spinal cord reserve, by confirming the association between CCaA, mainly measured at C3/C4 level, and disability progression. It also reveals differences in CCaA measurements among HC and people with MS phenotypes in a multicentric cohort. In particular, no differences in CCaA were observed between HC and PwRMS, a prerequisite to consider CCaA a valid proxy for spinal cord reserve. Conversely, progressive patients exhibited a smaller CCaA, suggesting that a lower spinal cord reserve might be a feature of progressive MS phenotype. Therefore, the spinal cord reserve may represent a novel radiological feature to better understand physical disability in MS.

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	C2/C3 Level				C3/C4 Level				Excluded subjects			
	HC N=135	PwRMS N=207	PwPMS N=97	[1]P value	HC N=142	PwRMS N=208	PwPMS N=100	[1]P value	HC N=45	PwRMS N=81	PwPMS N=40	[1,2]P value
Sex (female) n (%)	76 (56.3)	140 (67.6)	50 (51.5)	0.008	80 (56.3)	139 (66.8)	51 (51)	0.017	28 (62.2)	48 (59.2)	26 (0.65)	>0.05
Age [years] mean (SD)	40.4 (13.2)	42.1 (11.5)	55.2 (8.9)	<0.001	40.0 (13.2)	42.0 (11.5)	55.3 (9.3)	<0.001	38.6 (14.7)	43.9 (13.0)	53.0 (9.8)	>0.05
EDSS base-line p50 (IQR)	-	2.5 (1.5 – 3.5)	6 (5 – 6.5)	<0.001	-	2.5 (1.5 – 3.5)	6 (5 – 6.5)	<0.001	-	2.75 (1.5 – 4)	6 (4.75 – 6.75)	>0.05
D.D [years] mean (SD)	-	11.2 (9.1)	19.9 (9.1)	<0.001	-	11.1 (9.1)	20.02 (9.3)	<0.001	-	11.9 (8.0)	18.7 (11.9)	>0.05
Cord le-sions p50 (IQR)	-	2 (0 – 3)	3 (2 – 6)	<0.001	-	2 (0 – 3)	3 (2 – 6)	<0.001	-	2 (1 – 3)	4 (1 – 6)	>0.05
TIV [mL] mean (SD)	1468.9 (64.1)	1440.9 (78.9)	1374.7 (61.4)	0.006	1470.7 (64.9)	1439.3 (80.1)	1375.5 (64.3)	0.01	1495.2 (68.7)	1426.3 (75.3)	1373.5 (72.5)	>0.05
Brain T2LV [mL] mean (SD)	-	8.2 (10.0)	16.2 (15.6)	<0.001	-	8.2 (10.1)	16.5 (15.7)	<0.001	-	8.8 (11.2)	13.5 (10.4)	0.01

Table 1: Demographic, clinical and radiological characteristics of the studied cohort. [1]p values correspond to univariate comparisons using parametric and non-parametric tests, as convenience. [2]Data from excluded patients is compared to the C2/C3 cohort for each phenotype. D.D, disease duration; HC, healty controls; IQR, interquartile range; PwPMS, people with progressive multiple sclerosis; PwRMS, people with relapsing multiple sclerosis; p50, percentile 50 (median); SD, standard deviation; TIV, total intracranial volume; T2LV, T2-lesion volume. - Dash indicates not information available. Of note, relapsing MS includes clinically isolated syndrome and relapsing-remitting multiple sclerosis; progressive MS includes primary progressive and secondary progressive multiple sclerosis.

	C2/C3			C3/C4		
	EDSS			EDSS		
	Whole cohort	PwRMS	PwPMS	Whole cohort	PwRMS	PwPMS
CCaA	β−0.05	β−0.19***	β 0.22	β−0.13***	β−0.16*	β 0.11
Age	β 0.50***	β 0.48***	β -0.007	β 0.48***	β 0.45***	β -0.05
Sex (Male)	β 0.05	β 0.09	β -0.32	β 0.06	β 0.10	β -0.09
SC lesions	β 0.28***	β 0.16**	β 0.26*	β 0.26***	β 0.13*	β 0.04
SCPF	β -0.11*	β−0.02	β−0.07	β−0.05	β−0.07	β−0.07
Adjusted-R^2	0.44	0.35	0.10	0.43	0.33	0.01
Model p-value	<0.001	<0.001	0.03	<0.0001	<0.0001	0.32
Shapiro-Wilk test (CCaA)	0.001	0.17	0.11	0.12	0.37	0.89
Breusch-Pagan test	0.06	0.16	0.26	0.05	0.23	0.22
Collinearity (IF)	1.08	1.07	1.05	1.16	1.13	1.11

Table 2: Multivariate regression models to investigate the association between EDSS and Cervical Canal Area (CCaA) at baseline, measured at C2/C3 and C3/C4 intervertebral disc levels. The table shows adjusted beta coefficients for each variable in every single regression model. At each vertebral level, the linear models are built in three different ways: using the entire cohort, or only people with relapsing MS or progressive MS. EDSS represents the dependent variable. Assumptions of linear regression are also being appraised. CCaA, cervical canal area; EDSS, Expanded Disability Status Scale; IF, inflation factor; MS, multiple sclerosis; SC, spinal cord; SCPF, spinal cord parenchyma fraction; Significance of β coefficient: ***p<0.001, **p<0.01, *p<0.05

Figure Titles and Legends

Figure 1. Flow diagram of the studied cohort.

See Methods and Results sections for a detailed explanation of patients excluded and reasons for exclusion. C2/C3 and C3/C3 refer to the cervical intervertebral disc levels

Figure 2. Cervical canal area at C2/C3 and C3/C4 intervertebral disc levels according to the different phenotypes.

Dots represent individual values. White dots show the median, inner boxes represent Q1 and Q3, and vertical whiskers indicate Q3+/-1.5 IQR. P values were obtained in age- and sex-adjusted regression models (see main text). CCaA, cervical canal area; HC, healthy controls; MS, multiple sclerosis; Q1, first quartile; Q3, third quartile; IQR, interquartile range.

Figure 3. Exemplary case.

Qualitative differences in CCaA segmentation at C2/C3 intervertebral disc level (red) and at C3/C4 level (green). A: we observed an overestimation of the CCaA in the segmentation at this level. B: CCaA segmentation is more accurate at C3/C4 in the same subject. CCaA, cervical canal area