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“IN VIVO ASSESSMENT OF GLYMPHATIC SYSTEM IN MULTIPLE SCLEROSIS”

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1. Introduction

Multiple sclerosis (MS) is a chronic, debilitating disease and represents the major non-traumatic cause of neurological disability in young people. The disease usually starts with a course of recurrent and reversible neurological deficits, the so-called 'relapsing-remitting' MS (RRMS) disease course (1) in approximately 85% of MS patients. At this stage, focal areas of inflammatory demyelination in which myelin and axons are damaged cause reversible disability. About 20 years after disease onset, RRMS converts toward secondary progressive MS (SPMS) (2) characterised by progressive disability accrual with or without acute inflammatory events (1). Ten % of MS patients presents with a progressive disease course since disease onset defining the so-called primary progressive MS (PPMS) disease phenotype. From the pathological point of view, it is thought that whilst acute inflammation and demyelination underpins RRMS, progressive neuronal loss is the leading cause of disability accrual in progressive MS phenotypes (3). However, the exact molecular and cellular mechanisms fuelling both demyelination and neurodegeneration are still to be determined. Blood-brain barrier breakdown, focal leucocytes infiltration, microglia activation and the release of pro-inflammatory cytokines (i.e., tumor necrosis factor- α , interleukin-1 β , interferon- γ) contribute to focal demyelination within white matter areas and cortex (4). At same time, cytotoxic factors (i.e., glutamate and reactive oxygen species), leucocytes infiltrates, the disruption of the axon-myelin unit and retrograde degenerative processes produce neuronal loss at the level of cortical and deep gray matter structures (4, 5). Furthermore, mitochondrial dysfunction and reactive oxygen species production, increased intracellular sodium concentration, neuronal acidosis and accumulation of iron also play a detrimental role

in the complex puzzle of MS pathology ultimately resulting in disease-related clinical disability (3, 4). Therefore, considering the toxic effect of such waste products on tissue, to counteract for such pathological trigger, it is arguable that the central nervous system (CNS) should be equipped with a waste clearing system. Differently from peripheral tissues that are cleared through lymph vessels (6), the CNS has for long been thought to lack a conventional lymphatic system and was defined as an 'immune-privileged' site. However, accumulated evidence in the last decade has outlined the existence of a CNS 'waste clearance' system, defined as glymphatic system, from glia, contributing to the system functioning, and lymphatic vessels (7). Actually, this breakthrough discovery was not entirely novel since its first description dates back to the Italian anatomist Paolo Mascagni and prior reports within the last eight decades supporting evidence to the existence of a lymphatic system in the CNS already exist (8-13). Notwithstanding the lack of lymphatic vessels embedded within brain tissue, transfer and clearance of solutes from the cerebral interstitial extracellular space is performed through a continuous exchange of interstitial fluid (ISF) with cerebrospinal fluid (CSF). Specifically, CSF penetrates brain parenchyma through perivascular space around arteries crossing the blood-brain barrier and then enters the interstitial space through an astrocytic aquaporin 4-dependent process (7, 14). ISF moves toward perivenous spaces, enters the perivenous space and flows along with veins toward brain meninges where it is collected in meningeal lymphatic vessels located next to major venous sinuses (Fig. 1) (15-17). CSF and ISF, named as glymphatic fluid, collect and remove waste products while passing through CNS tissue (7) contributing to glymphatic system function. While the aforementioned hypothesis is attractive, available data about glymphatic anatomy and functioning in healthy controls (HCs) and neurological conditions are still preliminary.

Some scientists have recently questioned the extramural perivascular CSF efflux arguing that ISF is drained through the intramural periarterial drainage model (18, 19). Indeed, in both extra- and intra-mural perivascular models the drainage efflux pathway flows parallel to brain vessels.

Despite the acquired knowledge about glymphatic physiology and pathology, the *in vivo* assessment of glymphatic function is still limited by the lack of non-invasive imaging techniques for its quantification. Glymphatic system has been previously assessed *in vivo* using dynamic PET imaging (23) or isotope- and contrast-based imaging techniques in HCs and patients with several neurological conditions, including MS (15, 24-28). Recently, the diffusion along perivascular space (DTI-ALPS) index, a diffusion-weighted MRI-based index, has been proposed as a less invasive alternative method to evaluate glymphatic function (29). DTI-ALPS index provides a measure of diffusivity along the perivascular space by taking advantage of the perpendicularity condition between veins and, thus, perivascular space, and fibres direction adjacent to the lateral ventricles. By dividing diffusivity over the perivascular axis and diffusivity over the axes perpendicular to dominant fibres axes, namely the y-axis for projecting fibres and z-axis for the associative fibres, to take into account the difference of water diffusivity in the tissue over different directions, we could assess pathological changes involving specifically the perivascular space, providing a proxy for glymphatic system functioning (Fig. 2). Previous studies demonstrated that DTI-ALPS index reduces with aging (30) and is lower in several neurodegenerative conditions, such as Alzheimer's disease (29), idiopathic normal pressure hydrocephalus (31, 32) and Parkinson's disease (33) compared with HCs. However, no studies have been performed so far to assess glymphatic function in MS through the application of the DTI-ALPS index.

2. Aims

By using advanced MRI imaging, including diffusion and susceptibility-weighted images, we measured the DTI-ALPS in a cohort of MS patients (RRMS and progressive MS [PMS]) and we assessed correlation between the DTI-ALPS index, clinical disability and MRI measures of demyelination and neurodegeneration (focal lesions in the white matter and gray matter, microstructural normal-appearing white matter abnormalities and cortical and deep gray matter atrophy). In line with previous findings in other neurological conditions (29, 33, 34), we would expect lower DTI-ALPS index in MS patients *vs* HCs, associated with both neurodegenerative and demyelinating measures, with a direct impact on clinical outcomes.

3. Materials and methods

3.1 Subjects

In this study, we included HCs and patients diagnosed with MS following McDonald diagnostic criteria (35) satisfying the following inclusion criteria:

- 1) right-handedness (36), because DTI-ALPS is calculated through regions of interests (ROIs) drawn on the dominant hemisphere (29, 36);
- 2) no history of significant medical illnesses (no major cardiovascular disorders that may possibly impair cardiovascular brain impulses impacting glymphatic flux (37));
- 3) no other major systemic, psychiatric or neurological diseases;
- 4) no contraindications to MRI (i.e., claustrophobia, pregnancy or breastfeeding).

Patients had to be relapse- and steroid-free for at least three months before MRI acquisition and have a stable disease-modifying treatment from at least 6 months.

All MS patients underwent a clinical examination, including the assessment of the Expanded Disability Status Scale (EDSS) score (38). This study received approval from the local ethical standard committee on human experimentation. All subjects provided the written informed consent prior to study participation. The study was performed in accordance with good clinical practices and the Declaration of Helsinki.

3.2 MRI acquisition

MRI scan for each included subject was performed using a 3.0 T Philips Ingenia CX scanner (Philips Medical Systems) between 1 p.m. and 8 p.m., to minimize

intersubjects' differences associated with the sleeping status, since sleeping status was associated with glymphatic system efficiency (39, 40). The MRI protocol included the following sequences: (1) sagittal 3D fluid attenuated inversion recovery (FLAIR), field of view=256×256 mm, pixel size=1×1 mm, 192 slices, 1 mm-thick, matrix=256×256, repetition time=4800 ms, echo time=270 ms, inversion time=1650 ms, echo train length=167; (2) sagittal 3D T1-weighted magnetization-prepared rapid gradient-echo, field of view=256×256, pixel size=1×1 mm, 204 slices, 1 mm-thick, matrix=256×256, repetition time=7 ms, echo time=3.2 ms, inversion time=1000 ms, flip angle=8°; (3) axial pulsed-gradient spin echo single shot diffusion-weighted echo planar imaging; 3 shells at b-value=700/1000/2855 s/mm² along 6/30/60 non-collinear directions and 10 b=0 volumes were acquired, field of view=240×233 mm, pixel size=2.14×2.69 mm, 56 slices, 2.3 mm-thick, matrix=112×85, repetition time=5900 ms, echo time=78 ms and three additional b=0 volumes with reversed polarity of gradients for distortion correction; (4) sagittal 3D double inversion recovery, field of view=256×256 mm, pixel size=1.2×1.2 mm, 195 slices, 1.3 mm-thick, matrix=214×214, repetition time=5500 ms, echo time=254 ms, inversion times=1650/2550 ms, echo train length=173; (5) 3D susceptibility weighted image (SWI), field of view =230x230, pixel size=0.60x0.60 mm, 135 slices, 2 mm-thick, matrix=384x382, repetition time=39 ms, echo times=5.5:6:35.5 ms, flip angle=17°; both magnitude and phase images for each echo were saved.

3.3 MRI analysis

For each subject, we calculated the total T2-hyperintense lesion volume by defining white matter lesions through a fully automated approach based on two 3D patch-wise convolutional neural networks, using 3D FLAIR and 3D T1-weighted MRI sequences (41). Cortical lesions were identified on double inversion recovery images including both cortical lesions confined to the cortical ribbon without involving the underlying subcortical white matter and mixed white/gray matter lesions with a predominant (>50%) extension in the cortex (42). We also calculated normalized cortical gray matter volume using FMRIB Software Library (FSL) SIENAx software on the lesion-filled 3D T1-weighted images (43) and normalized deep gray matter volume by summing the volumes of the thalamus, caudate, putamen, pallidum, amygdala and nucleus accumbens, obtained by segmenting these structures on the lesion-filled 3D T1-weighted images through the FIRST tool from the FSL (44).

Concerning diffusion-weighted images, we performed correction for off-resonance and eddy current induced distortions, for slice-to-volume and overall movements and for outliers by applying the Eddy tool within the FSL (45). Using diffusion-weighted imaging data at $b=700$ and 1000 s/mm^2 we estimated diffusion tensor by linear regression and we calculated eigenvalues, eigenvectors, maps of fractional anisotropy (FA) and mean diffusivity (MD) after diagonalization of the diffusion tensor matrix (46).

To obtain a subject-specific map of brain venous vessels, we derived local B_0 field changes images from the multi echo SWIs using <https://github.com/sunhongfu/QSM>. Phase images were unwrapped to eliminate discontinuities due to the limited range of

phase values, using the best path method (47). Unwrapped phase images were subsequently fit to the echo time, using a magnitude-weighted least square regression. Finally, we removed global spatial changes of the main magnetic field to increase the visibility of small brain veins (48).

3.4 DTI-ALPS quantification

DTI-ALPS index is calculated following steps depicted in Fig. 3. In detail, FA color map and T2 lesion mask for each subject were registered in the SWI space using the magnitude of the first echo of the SWI sequence as reference image using FLIRT (FMRIB's Linear Image Registration Tool) embedded in the FSL. 'Vecreg' utility from the FSL and the rigid transformation matrix previously obtained were subsequently acquired to register the principal diffusion direction field images to the SWI images (49). Using venous vessel images for each subject we selected the three contiguous axial slices where veins run perpendicular to the lateral ventricle and, hence, perivascular spaces, run over the x-axis. Over these slices, we drew two 3x3x3mm cubic ROIs using the color-coded principal diffusion direction map: one ROI was drawn over the area of projection fibers (ROI_{proj}) and the other over the area of associative fibers (ROI_{assoc}). We drew ROIs in the left hemisphere and we enrolled only right-handed subjects to be sure that the associative fibers, namely the superior longitudinal fasciculus, are thick enough to minimize the possibility of losing the perpendicularity between fibers axis and perivascular space (29). T2 lesion mask for

each subject was used to avoid drawing ROIs over damaged tissue. ROIs were registered to the diffusion imaging space by applying the inverse diffusion-to-SWI transformation; all ROIs were visually inspected and voxels not falling within either projecting or associative fibers for ROI_{proj} and ROI_{assoc}, respectively, were discarded. After inspection, no corrections were needed. Finally, diffusivity along the x-, y, and z- axes was extracted for each ROI.

DTI-ALPS index was calculated using the following formula:

$$DTI - ALPS = \frac{\text{mean}(Dx \text{ proj}, Dx \text{ assoc})}{\text{mean}(Dy \text{ proj}, Dz \text{ assoc})}$$

3.5 Statistical analysis

Statistical analyses were performed using Stata software (version 13; StataCorp LP, College Station, TX). Demographic, clinical and MRI features are presented as means, medians or proportions and standard deviation (SD) or ranges. Normal distribution for continuous variables was explored either through Shapiro-Wilk test or graphical approaches. Cortical and white matter lesions were log-transformed to ensure Gaussian distribution. T-Test, Mann–Whitney U or Chi-squared tests were employed to assess between-group comparisons of demographic, clinical and structural MRI features. To assess DTI-ALPS index reproducibility, we performed an inter- and intra-rater analysis by ROIs drawing from two raters or from the same rater 30 days apart in 5 MS patients and 5 HCs. Intraclass correlation coefficient was calculated and labeled as ‘almost perfect’ if higher than 0.81 (50).

To analyze differences between MS patients and HCs for MRI measures, including the DTI-ALPS index, taking into account the impact of aging and sex- on DTI-ALPS index (30) and MRI measures (51), we performed multiple linear regression models adjusted for age- and sex and analysis was adjusted for multiple comparison through the false discovery rate (FDR) correction method. Between-groups estimated mean differences and 95% confidence intervals are reported.

Pair-wise correlations and FDR correction were applied to evaluate correlation between DTI-ALPS, clinical disability (assessed with the EDSS), disease duration, white matter and cortical lesion volumes, cortical and deep gray matter volumes, mean FA and MD in MS patients and between DTI-ALPS index and cortical and deep gray matter volumes, mean FA and MD in HCs. Correlation between disease duration and DTI-ALPS index was further explored through a "hockey stick" regression with the search of the breakpoint to indirectly assess changes of glymphatic function over MS course (52). FDR-adjusted p values lower than 0.05 were considered statistically significant.

4. Results

4.1 Clinical Assessments

We enrolled 71 MS patients (mean age=43.5 years, SD=10.3 years; 30/41 men/women, 49 relapsing-remitting MS [RRMS] and 22 progressive MS [PMS] patients (53)) and 32 HCs (mean age=42.2 years, SD=11.1 years; 18/14 men/women). HCs and MS patients were comparable for age and sex. Compared with RRMS, PMS patients were significantly older, had higher EDSS score, longer disease duration, higher white matter and cortical lesion volume and a higher proportion of patients with at least one cortical lesion ($p < 0.01$ for all comparisons).

Clinical, demographic and imaging characteristics of the HCs and MS patients are summarized in Table 1.

4.2 DTI-ALPS index: inter- and intra- rater correlation

To assess overlap between T2 lesions and ROIs we drew probability maps of white matter lesions and ROIs. Results are shown in Fig. 4. There was no overlap between ROI_{proj} and ROI_{assoc} . Conversely, we observed a partial overlap between white matter lesions and ROI_{proj} . Intraclass correlation coefficient for inter-rater correlation was 0.92 being a mean DTI-ALPS from rater 1 of 1.63 ± 0.32 and a mean DTI-ALPS from rater 2 of 1.67 ± 0.25 . Similarly, intraclass correlation coefficient for intra-rater correlation was 0.93 with the first DTI-ALPS measure being 1.63 ± 0.32 and the second 1.63 ± 0.29 .

4.3 Multiple sclerosis patients versus HCs

Compared with HCs, MS patients showed lower cortical (estimated mean difference [EMD]: -15.68, $p=0.04$) and deep gray matter (EMD: -3.39, $p=0.009$) volume, lower mean FA (EMD: -0.05, $p=0.001$), higher mean MD (EMD: 0.04, $p=0.001$) and lower DTI-ALPS index (EMD: -0.09, $p=0.01$, Fig. 5) (Table 2). Dividing MS patients according to disease phenotype we reported lower deep gray matter volume (EMD: -2.80, $p=0.02$), lower mean FA (EMD: -0.04, $p=0.006$), higher mean MD (EMD: 0.03, $p=0.001$) and lower DTI-ALPS index (EMD: -0.06, $p=0.04$, Figure 5) in RRMS vs HCs and lower cortical (EMD: -26.81, $p=0.006$) and deep gray matter (EMD: -5.93, $p=0.001$) volume, lower mean FA (EMD: -0.08, $p=0.001$), higher mean MD (EMD: 0.06, $p=0.001$) and lower DTI-ALPS index (EMD: -0.19, $p=0.001$, Figure 5) in PMS patients vs HCs. Compared with RRMS patients, PMS patients showed lower mean FA (EMD: -0.04, $p=0.05$) and lower DTI-ALPS index (EMD: -0.09, $p=0.03$, Figure 5).

4.4 Correlation of DTI-ALPS index and clinical/MRI measures

In HCs, we showed that higher DTI-ALPS index correlated with higher mean FA ($r=0.55$, $p=0.002$) and lower mean MD ($r=-0.36$, $p=0.02$). Similarly, in MS patients, lower DTI-ALPS index correlated with higher mean MD ($r=-0.45$, $p=0.001$), lower mean FA ($r=0.45$, $p=0.001$). In addition, in MS patients lower DTI-ALPS index also correlated with higher EDSS ($r=-0.45$, $p=0.001$), longer disease duration ($r=-0.37$, $p=0.002$), higher white matter ($r=-0.36$, $p=0.003$) and cortical ($r=-0.41$, $p=0.001$) lesion volume, and lower cortical ($r=0.30$, $p=0.007$) and deep gray matter ($r=0.42$, $p=0.001$) volume. Results from correlation analyses are shown in Table 3. Finally, piece-wise correlation analysis showed that lower DTI-ALPS index correlated with longer disease

duration up to 4.13 years from disease onset ($r=-0.38$, $p=0.04$). After this breakpoint, DTI-ALPS index was no longer correlated with disease duration.

5. Discussion

The complex interplay between ISF-CSF solute exchange, facilitated by the spaces surrounding intracerebral blood vessels or the blood vessel walls and dural lymphatics, comprehensively embedded in the concept of ‘glymphatic system’, has received a growing interest in the last decade. Whilst the interest for studying glymphatic in healthy subjects as well as in neurodegenerative conditions has dramatically surged, MRI assessment of glymphatic function is still hampered by the lack of direct, specific and non-invasive imaging tools. Initial MRI reports about glymphatic functioning required either induced blood–brain barrier opening to let contrast injected intravenously to enter the glymphatic space (27) or intrathecal contrast injection (25). However, the invasive nature of these MRI techniques prevents scientists from a wider application of such methods and, thus, to assess glymphatic function in different neurological conditions.

To overcome this limitation, diffusion-weighted imaging allowing calculation of the DTI-ALPS index, was proposed as an indirect measure of glymphatic function, at least for what it concerns its perivascular component (29). In our study, we demonstrated that DTI-ALPS index showed a high inter- and intra-rater correlation coefficient, with an ‘almost perfect’ agreement (intraclass correlation coefficient >0.81). Our finding adds confirmation to previously reported results about DTI-ALPS index reliability and reproducibility (50) confirming that this index can be applied from different rater and under different MRI parameters without impacting its reproducibility. However, since the proper calculation of DTI-ALPS index relies on the assumption of perpendicularity between white matter fibers and veins direction, in a CNS

disorders characterized by the random location of white matter demyelinating lesions mining this perpendicularity it is highly needed to assess the possible automatic ROI setting in MS, as it was previously proposed in Parkinson's and Alzheimer's disease (32). To this aim, we evaluated ROIs probability maps together with white matter lesion probability maps. Although there was no overlap between ROIs, we detected an overlap between ROI_{proj} and lesions in periventricular areas. Therefore, despite automatic ROI drawing being preferred over manually defined ROIs in neurodegenerative disorders (32), our study supports the need for manually defined ROIs in MS.

In line with this *caveat*, we explored glymphatic function in MS by calculating the DTI-ALPS index in MS patients and HCs using manually defined ROIs. Overall, we reported on impaired glymphatic function in MS patients compared with HCs. Such an impairment was previously suggested in MS experimental models, where venous-lymphatic interface was pointed as the area where patrolling resident lymphocytes are activated from antigen-presenting cells toward a pro-inflammatory and demyelinating configuration (34). Lymphatic vessels collect glymphatic fluid from brain parenchyma and drain this fluid in the cervical lymph nodes contributing to the overall glymphatic system proper function. Therefore, glymphatic system damage may promote inflammatory activation by altering lymphatic vessels-dependent CNS immune surveillance. Beyond its function at cortical regions, given that ISF-CSF solute exchange occurs in perivenous spaces within the brain tissue before flowing into lymphatic vessels (7), we may also hypothesize that a slower flow in perivascular space might promote the accumulation of pro-inflammatory molecules in the close proximity of venous wall, triggering demyelination. This could also match with the so-called 'central vein' sign in MS,

which is the presence of a venous vessel within white matter demyelinating lesions (54). In humans, to date, only one study explored glymphatic function in a small sample of RRMS patients using PET. Authors showed an impaired CSF clearance in patients compared with HCs (23). Given the small sample size and the exclusively inclusion of RRMS patients, this PET study requires confirmation. Our study, including a larger cohort of MS patients spanning the remitting and progressive phenotypes, confirmed that glymphatic system is impaired in MS, with a prominent impairment in progressive MS phenotype. While preliminary evidence already exists for glymphatic system impairment in RRMS patients (23), glymphatic function was not hitherto investigated in PMS. In line with our results we may speculate that glymphatic system undergoes a progressive impairment since earliest disease stages becoming severely affected later over the disease course. Indeed, the piece-wise correlation analysis between the DTI-ALPS index and disease duration provided a clue for hypothesizing that glymphatic function worsens over the first 4 years from disease onset. Therefore, DTI-ALPS index may represent a marker of 'below-the-radar' disability progression and might be possibly used to identify those MS patients with the highest risk of conversion toward progressive stages. Furthermore, such hypothesis is corroborated by the fact that in MS experimental models parenchymal CSF circulation alteration mirrors the temporal and spatial occurrence of MS disease onset (55). In details, the co-occurrence of the two phenomenon (i.e., CSF alteration and acute neuroinflammation) may be due to the infiltration of the immune cells in the perivascular space altering the proper glymphatic functioning or, *vice versa* to the impairment of CSF circulation promoting immune infiltration. The dynamic between glymphatic impairment, neuroinflammation at disease onset and progressive neurodegeneration requires

further investigation with pathology and prospective longitudinal studies. The extent of glymphatic system impairment may reflect, hence, underlying accumulation of CNS tissue damage ultimately resulting in clinical disability. The only available study assessing the correlation between glymphatic impairment and clinical disability in MS found no association (23). However, this study had a limited sample size, including only 19 RRMS patients, who might have a more limited range of EDSS span comparing with our study. Conversely, in other neurodegenerative disorders such as Alzheimer's and Parkinson's disease there was a strong association between clinical disability and glymphatic impairment (29, 33, 56, 57). In the search of pathological substrates associated with glymphatic impairment underpinning disability burden, we performed a correlation analysis between MRI measures of tissue integrity and DTI-ALSP index and we observed that impaired glymphatic system function correlated with neuronal loss at cortical and subcortical areas and the total volume of demyelinating lesions (both at cortical and white matter level). To date there is no clear link about these pathological processes. However, taking into account that DTI-ALPS index is calculated in ROIs placed close proximity to the lateral ventricles it may actually reflect removal of toxic molecules from deep gray matter nuclei. A gradient of gray matter damage from ventricles/deep gray matter nuclei surface outward was already reported in MS from the very early stages of the disease (58). Therefore, glymphatic impairment may cause gray matter pathology by not removing CSF-derived toxic molecules drained in perivascular spaces in deep gray matter area. Similarly, the reduced glymphatic fluid flow in the perivenous spaces may cause the accumulation of neuroinflammatory triggers in the white matter, thus leading to demyelinating processes and a reduced *momentum* for flow diffusion in meningeal lymphatic

vessels, leading to accumulation of inflammatory and neuro-toxic elements, such as meningeal T cells and toxic cytokines (e.g., the chemokine ligand 13, the interferon- γ , the tumor necrosis factor and lymphotoxin- α/β), contributing to cortical demyelination (59). Therefore, glymphatic impairment may cause MS-related pathology by accumulating toxic molecules leading to an increased inflammatory status and detrimental neurotoxic stimuli. Ultimately, we also demonstrated an association between glymphatic impairment and microstructural tissue damage in the normal-appearing white matter (i.e. reduced FA and increased MD). It would be interesting to evaluate whether damages to normal appearing white matter would be the cause or the result of glymphatic impairment. I.e. MS demyelination could affect localization and polarization of aquaporin-4 at the end feet of the astrocytes, thus impairing glymphatic fluid circulation by disrupting the perivascular space-interstitial barrier. Morphological changes has been already described for astrocytes falling within acute demyelinating lesions, with an enlargement of the soma and a reduced feet density (60).

Notwithstanding the novelty of this study, we should outline a few limitations. First, the dynamic interplay between glymphatic damages, neurodegeneration and neuroinflammation could not be completely unveiled given the cross-sectional nature of the study. Longitudinal studies may also eventually evaluate if glymphatic function could predict disease conversion from relapsing to progressive stages over the disease course.

Second, DTI-ALPS index is an indirect method to assess glymphatic system by measuring diffusivity within perivascular spaces at deep gray matter areas and, hence, not completely reflecting the overall glymphatic function. In order to comprehensively evaluate glymphatic function, a multi-parametric study, applying contrast-based dynamic approaches

and DTI-ALPS index, may further elucidate and validate this measure against the function of the whole glymphatic system.

6. Conclusions

DTI-ALPS index calculated through a non-invasive diffusion-based imaging approach could be used as a proxy for glymphatic system functioning. Using this measure, we showed that MS patients experience impairment in the glymphatic system at both RRMS and PMS stages, being more severely affected in PMS. We also demonstrated that reduced glymphatic function was also associated with a more severe clinical disability and other MRI markers of disease pathology.

Overall, our findings point toward the possibility that glymphatic system impairment contributes to MS pathology ultimately resulting in disability accrual.

7. Figures

Figure 1.

Glymphatic schematic picture. From arteries, cerebrospinal fluid crosses the perivascular space entering the interstitium and moving toward perivenous spaces. While moving, fluid collects toxic molecules and cells cleaning up brain parenchyma. Once in the perivenous space, the fluid moves toward brain convexity where it is collected in the lymphatic meningeal vessels. Image from Natale G. et al. 2021 (61).

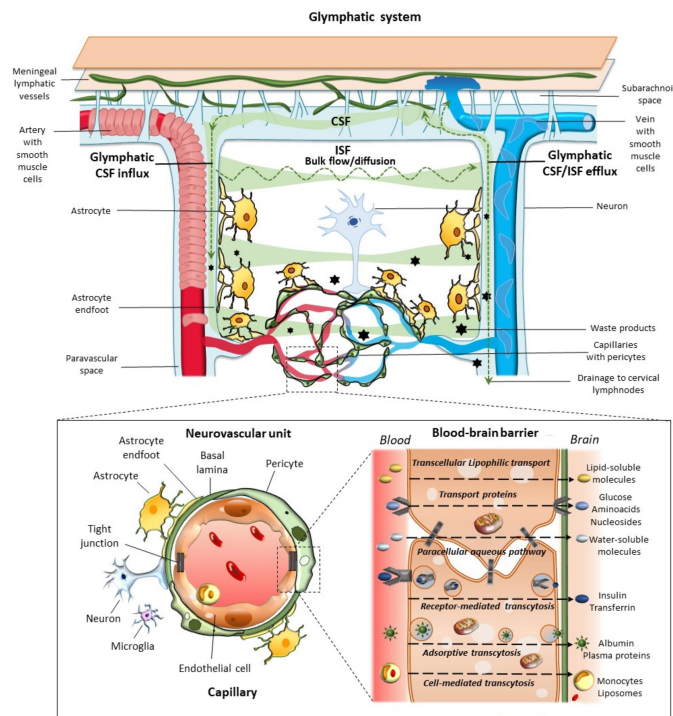


Figure 2.

Glymphatic physiology and regions of interests for DTI-ALPS index calculation.

Schematic picture of glymphatic anatomy and the position for the ROI over projection fibers and for the ROI over associative fibers to calculate DTI-ALPS. Image from Carotenuto et al. 2021 (62).

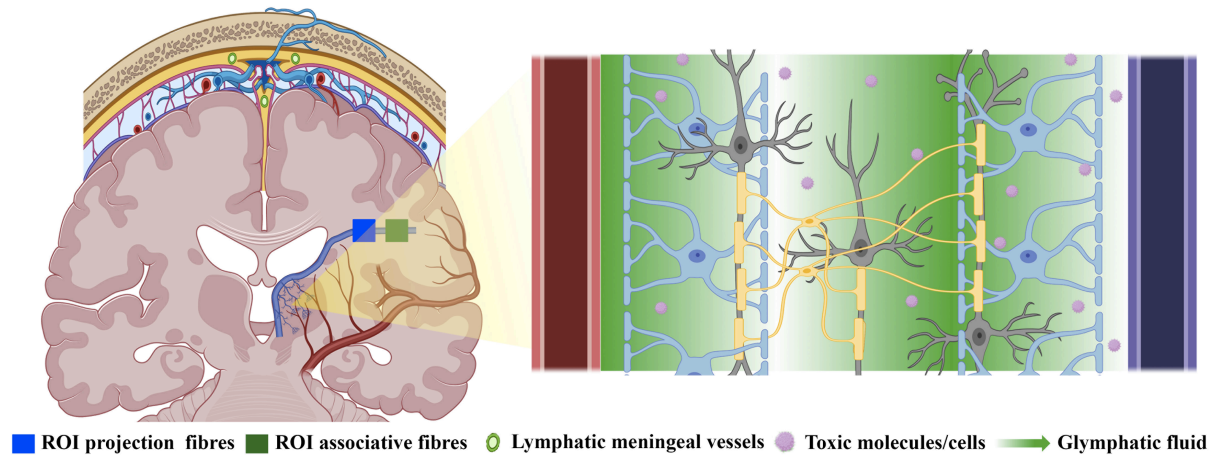


Figure 3.
DTI-ALPS quantification.

(A) 3D FLAIR, T2 lesion mask and FA map for each subject are firstly registered to the SWI scans. (B) Using vein images, we selected three slices among those available characterized by the major perpendicularity condition between and lateral ventricle. Over these slices, using the color-coded FA map in SWI space, two 3x3x3mm regions of interests (ROIs) were drawn: one over the area of projection fibers and the other over the area of associative fibers in the left hemisphere. We moved back ROIs to the diffusion space using the inverse of the FA map to-SWI registration matrix. (C) Finally, we extracted diffusivity values along the x-, y-, and z- axes for each ROI and we calculated the DTI-ALPS index as the ratio of diffusivities perpendicular to fibre bundles and parallel to veins (D_x proj and D_x assoc) over diffusivities perpendicular to fibre bundles and perpendicular to veins (D_y proj and D_z assoc). Image from Carotenuto et al. 2021 (62).

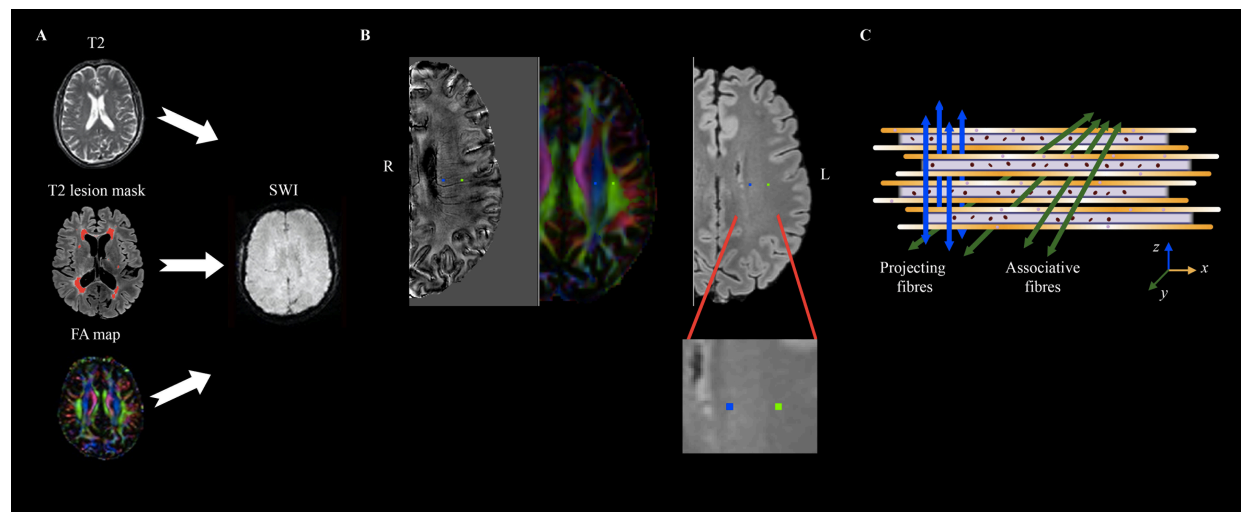


Figure 4.

Probability maps for white matter lesions, ROI_{proj} e ROI_{assoc}.

Spatial probability maps (thresholded at 25%) of all white matter lesions (red-yellow) and ROIs over projecting fibers (blue-light blue) and ROIs over associative fibers (green-light green) superimposed onto Montreal Neurological Institute (MNI) template. Probability intensity bars are reported as %. Image from Carotenuto et al. 2021 (62).

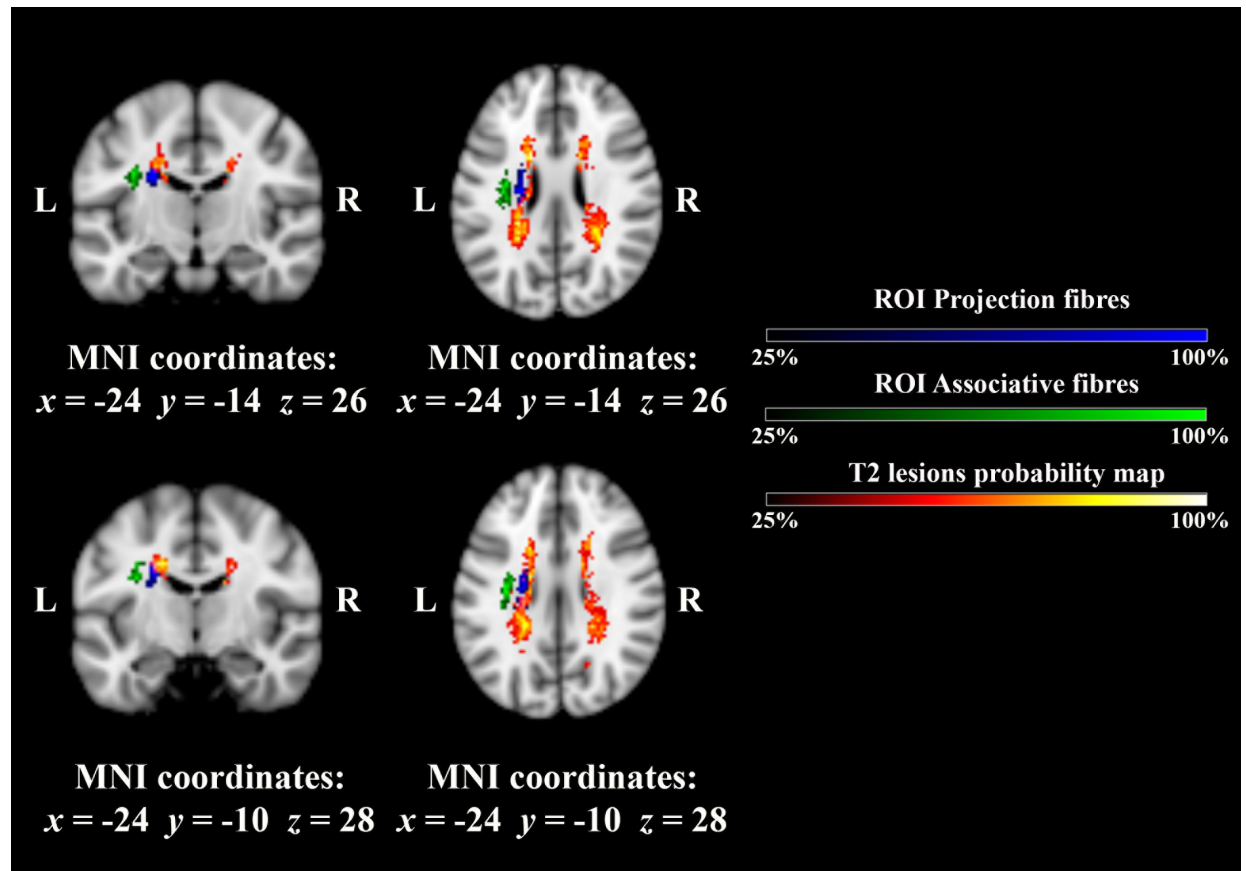
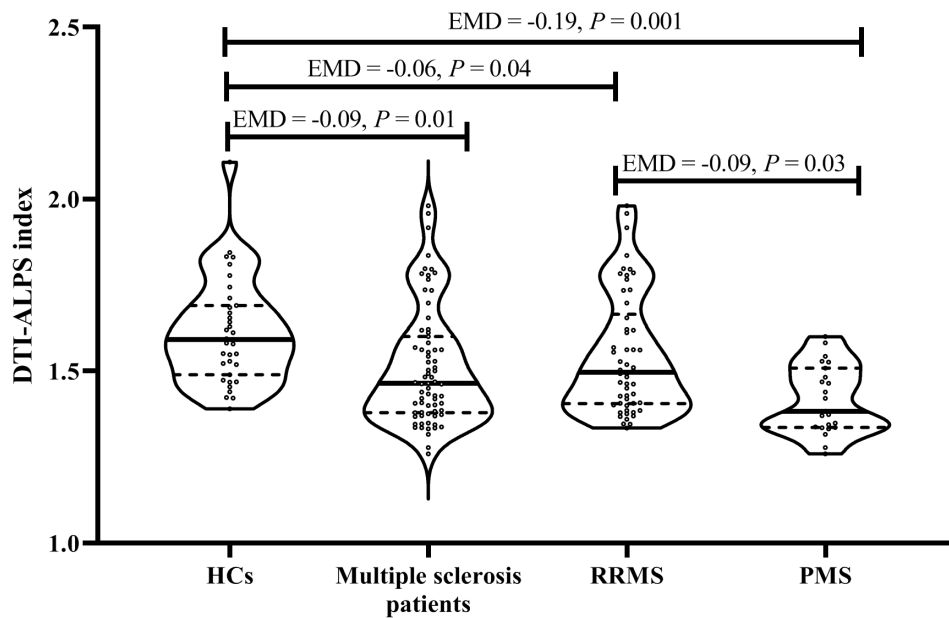


Figure 5.

Violin plot for DTI-ALPS index in HCs, multiple sclerosis patients, relapsing-remitting and progressive multiple sclerosis patients.

Estimated mean difference (EMD) and false discovery rate adjusted p values obtained through the between-groups comparison are shown. Image from Carotenuto et al., 2021 (62).



8. Tables

Table 1. Demographic, clinical and radiological features of MS patients and HCs.

	HCs	Multiple sclerosis	p value*	RRMS	PMS	p-value**
Subjects	32	71		49	22	
Sex	Female, N (%)	14 (4)	0.19 ^a	26 (53)	15 (68)	0.23 ^a
	Male, N (%)	18 (56)		23 (47)	7 (32)	
Age, mean $\pm$ SD (years)	42.2 $\pm$ 11.2	43.5 $\pm$ 10.3	0.40 ^b	41 $\pm$ 9.8	49.1 $\pm$ 9.1	0.007 ^b
EDSS, median (Range)	-	2.0 (0.0 – 7.0)	-	1.5 (0.0 - 4.5)	6.5 (4.0 – 7.0)	<0.001 ^c
Disease Duration, median (Range) (years)	-	5.5 (0 - 34)	-	3 (0 - 34)	17 (5 - 33)	<0.001 ^c
White matter lesion volume, mean $\pm$ SD (mL)	-	3.3 $\pm$ 0.6	-	3.16 $\pm$ 0.5	3.63 $\pm$ 0.58	0.002 ^b
Patients with cortical lesions, N (%)[§]	-	46 (66)	-	25 (51)	21 (100)	<0.001 ^a
Cortical lesion volume, mean $\pm$ SD (mL)[§]	-	1.3 $\pm$ 1	-	0.93 $\pm$ 1	2.24 $\pm$ 0.4	<0.001 ^b

*Multiple sclerosis patients vs HCs; ** PMS vs RRMS

^a Chi-square test; ^b t-Test; ^c Mann-Whitney U

[§]One multiple sclerosis patient missed double inversion recovery sequence.
Cortical and white matter lesion volume reported here were log-transformed.

Abbreviations: HCs=healthy controls; RRMS=relapsing-remitting multiple sclerosis; PMS=progressive multiple sclerosis; EDSS= Expanded disability status scale; SD=standard deviation.

Table 2. Between-group comparisons for MRI findings.

	HCs	Multiple sclerosis	Estimated mean difference [95%CI]	Multiple sclerosis vs HCs, p value	RRMS	Estimated mean difference vs HCs [95%CI]	RRMS vs HCs, p value	PMS	Estimated mean difference vs HCs [95%CI]	PMS vs HCs, p value
Cortical gray matter volume, mean (SD) mL	658 (24)	636 (52)	-15.68 [-36.5;5.1]	0.04*	643 (59)	-13.18 [-36.5;10.2]	0.06	623 (34)	-26.81 [-45.7;-7.9]	0.006*
Deep gray matter volume, mean (SD) mL	56 (3)	52 (6)	-3.39 [-5.9;-0.8]	0.009*	53 (6)	-2.80 [-5.41;-0.18]	0.02*	49 (5)	-5.93 [-8.86;-3]	0.001*
FA, mean (SD)	0.49 (0.04)	0.44 (0.07)	-0.05 [-0.07;-0.02]	0.001*	0.46 (0.07)	-0.04 [-0.06;-0.01]	0.006*	0.41 (0.06)	-0.08 [-0.12;-0.05]	0.001*
MD, mean (SD)	0.68 (0.02)	0.72 (0.05)	0.04 [0.02;0.05]	0.001*	0.71 (0.04)	0.03 [0.01;0.04]	0.001*	0.74 (0.07)	0.06 [0.03;0.09]	0.001*
ALPS Index, mean (SD)	1.61 (0.20)	1.51 (0.17)	-0.09 [-0.17;-0.02]	0.01*	1.56 (0.18)	-0.06 [-0.15;0.02]	0.04*	1.41 (0.1)	-0.19 [-0.29;-0.09]	0.001*

*Age- and sex-adjusted linear regression models. P values were corrected for multiple comparisons with false discovery rate.

Abbreviations: HCs=healthy controls; RRMS=relapsing-remitting MS; PMS=progressive MS; MD= Mean diffusivity; FA=fractional anisotropy; SD=standard deviation; CI=Confidence Interval.
Note: MD is expressed in units of $\text{mm}^2 \text{s}^{-1} \times 10^{-3}$, FA is a dimensionless quantity

Table 3. Correlations between DTI-ALPS index, EDSS, disease duration and structural MRI features.

		HCs	Multiple sclerosis
EDSS	Sperman's R	-	-0.45
	p value	-	0.001*
Disease duration	Sperman's R	-	-0.37
	p value	-	0.002*
White matter lesion volume	Sperman's R	-	-0.36
	p value	-	0.003*
Cortical lesion volume	Sperman's R	-	-0.41
	p value	-	0.001*
Cortical gray matter volume	Sperman's R	-0.03	0.30
	p value	0.08	0.007*
Deep gray matter volume	Sperman's R	0.19	0.42
	p value	0.09	0.001*
FA	Sperman's R	0.55	0.45
	p value	0.002*	0.001*
MD	Sperman's R	-0.36	-0.45
	p value	0.02*	0.001*

*Pair-wise correlation adjusted for multiple comparisons (false discovery rate [FDR]).

Abbreviations: DTI-ALPS=diffusion tensor image analysis along the perivascular space; HCs=healthy controls; EDSS= Expanded disability status scale; FA=Fractional anisotropy; MD= Mean diffusivity.

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