


## RESEARCH ARTICLE

# A “glympse” into neurodegeneration: Diffusion MRI and cerebrospinal fluid aquaporin-4 for the assessment of glymphatic system in Alzheimer's disease and other dementias

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## Abstract

The glymphatic system (GS) is a whole-brain perivascular network, consisting of three compartments: the periarterial and perivenous spaces and the interposed brain parenchyma. GS dysfunction has been implicated in neurodegenerative diseases, particularly Alzheimer's disease (AD). So far, comprehensive research on GS in humans has been limited by the absence of easily accessible biomarkers. Recently, promising non-invasive methods based on magnetic resonance imaging (MRI) along with aquaporin-4 (AQP4) quantification in the cerebrospinal fluid (CSF) were introduced for an indirect assessment of each of the three GS compartments. We recruited 111 consecutive subjects presenting with symptoms suggestive of degenerative cognitive decline, who underwent 3 T MRI scanning including multi-shell diffusion-weighted images. Forty nine out of 111 also underwent CSF examination with quantification of CSF-AQP4. CSF-AQP4 levels and MRI measures—including perivascular spaces (PVS) counts and volume fraction (PVSVF), white matter free water fraction (FW-WM) and mean kurtosis (MK-WM), diffusion tensor imaging analysis along the perivascular spaces (DTI-ALPS) (mean, left and right)—were compared among patients with AD ( $n = 47$ ) and other neurodegenerative diseases ( $n_{AD} = 24$ ), patients with stable mild cognitive impairment (MCI = 17) and cognitively unimpaired (CU = 23) elderly people. Two runs of analysis were conducted, the first including all patients; the second after dividing both  $n_{AD}$  and AD patients into two subgroups based on gray matter atrophy as a proxy of disease stage. Age, sex, years of education, and scanning time were included as confounding factors in the analyses. Considering the whole cohort, patients with AD showed significantly higher levels of CSF-AQP4 ( $\exp(b) = 2.05$ ,  $p = .005$ ) and FW-WM FW-WM ( $\exp(b) = 1.06$ ,  $p = .043$ )

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than CU. AQP4 levels were also significantly higher in nAD in respect to CU ( $\exp(b) = 2.98, p < .001$ ). CSF-AQP4 and FW-WM were significantly higher in both less atrophic AD ( $\exp(b) = 2.20, p = .006$ ;  $\exp(b) = 1.08, p = .019$ , respectively) and nAD patients ( $\exp(b) = 2.66, p = .002$ ;  $\exp(b) = 1.10, p = .019$ , respectively) compared to CU subjects. Higher total ( $\exp(b) = 1.59, p = .013$ ) and centrum semiovale PVS counts ( $\exp(b) = 1.89, p = .016$ ), total ( $\exp(b) = 1.50, p = .036$ ) and WM PVSVF ( $\exp(b) = 1.89, p = .005$ ) together with lower MK-WM ( $\exp(b) = 0.94, p = .006$ ), mean and left ALPS ( $\exp(b) = 0.91, p = .043$ ;  $\exp(b) = 0.88, p = .010$  respectively) were observed in more atrophic AD patients in respect to CU. In addition, more atrophic nAD patients exhibited higher levels of AQP4 ( $\exp(b) = 3.39, p = .002$ ) than CU. Our results indicate significant changes in putative MRI biomarkers of GS and CSF-AQP4 levels in AD and in other neurodegenerative dementias, suggesting a close interaction between glymphatic dysfunction and neurodegeneration, particularly in the case of AD. However, the usefulness of some of these biomarkers as indirect and standalone indices of glymphatic activity may be hindered by their dependence on disease stage and structural brain damage.

#### KEYWORDS

Alzheimer disease, aquaporin 4, brain perivascular spaces, cerebrospinal fluid, dementia, diffusion magnetic resonance imaging, glymphatic system, neurodegenerative disease

#### Practitioner Points

- Diffusion MRI and the quantification of AQP4 in the cerebrospinal fluid may serve as indirect biomarkers of glymphatic activity in vivo.
- Neurodegeneration is associated with diffuse changes of MRI parameters of glymphatic activity and AQP4 levels in the CSF.
- Changes of glymphatic biomarkers are partly depending on disease stage and measures of structural brain damage.

## 1 | INTRODUCTION

The glymphatic system (GS) is a perivascular network which plays a fundamental role in waste removal from the brain. GS has a three-compartment organization, in which subarachnoid cerebrospinal fluid (CSF) enters the brain's interstitium from the periarterial spaces facilitated by aquaporin-4 (AQP4), mixes with the interstitial fluid (ISF) and waste solutes—including pathological proteins like amyloid- $\beta$  ( $A\beta$ ) and tau—and is then drained out of the brain by perivenous efflux routes into meningeal and cervical lymphatics (Bohr et al., 2022; Iliff et al., 2012; Rasmussen et al., 2018).

Gold-standard in-vivo evaluation tools for GS require intrathecal (Zhang et al., 2021; Zhou et al., 2020) or intravenous (Zhang, Tang, et al., 2023) administration of gadolinium based contrast agents (GBCA) or radioactive tracers (Schubert et al., 2019), whose relative invasiveness has limited comprehensive research in humans. Recently, there has been a growing effort to identify less invasive and more easily accessible biomarkers of GS function.

Promising magnetic resonance imaging (MRI)-based techniques have been introduced for an indirect evaluation of glymphatic activity.

These include enlarged perivascular spaces (PVS) quantification and diffusion-based techniques.

PVS are fluid-filled compartments enveloping brain penetrating arterioles and are considered as a major component of the GS. PVS are known to become enlarged in many clinical conditions. Enlarged PVS in the basal ganglia (BG) are primarily associated with aging and hypertension (Bown et al., 2022; Evans et al., 2023), while enlarged PVS in the centrum semiovale (CSO) have been associated with amyloid deposition (Charidimou et al., 2014; Kim et al., 2021; Perosa et al., 2022) and neurodegeneration. Indeed, impaired glymphatic clearance may limit effective removal of pathological proteins from the brain and clog up the system upstream, leading to further reduction of perivascular drainage, PVS enlargement and ultimately neurodegeneration. Once dilated, PVS can be quantified on MRI using visual scoring scales (Paradise et al., 2020, 2021) or, more accurately, with a wide range of segmentation techniques, spanning from classical image processing approaches to deep neural network modeling (Ballerini et al., 2018; Barisano et al., 2022; Pham et al., 2022; Sepehrband et al., 2019).

Diffusion-weighted (DW)-MRI enables the evaluation of brain microstructure by probing microscopic diffusivity of water molecules (Bergamino et al., 2021). Most commonly, diffusion-related parameters are derived using the diffusion tensor (DT) model (i.e., DT-imaging: DTI) (Pierpaoli et al., 1996). The DTI-based analysis along the perivascular spaces (ALPS) was developed to evaluate the diffusivity along the PVS of the deep medullary veins, which is regarded as a proxy measure of glymphatic clearance (Taoka et al., 2017). The usefulness of ALPS index as a non-invasive biomarker of GS function was supported by a recent comparison with data obtained by intrathecal GBCA tracers (Zhang et al., 2021).

DT is a widely acknowledged model that, however, suffers from several limitations (Jones & Cercignani, 2010). To overcome these limitations, a free-water (FW) correction algorithm was developed to remove the contribution of extracellular FW to DTI-derived metrics (Pasternak et al., 2009). Elevated FW in the white matter (WM) has been observed in patients with AD, suggesting impaired fluid drainage likely attributable to glymphatic dysfunction (Dumont et al., 2019; Ji et al., 2017; Kamagata et al., 2022; Maier-Hein et al., 2015), and the FW index has been proposed as a standalone biomarker of AD-related pathology (Bergamino et al., 2021; Ji et al., 2017; Kamagata et al., 2022; Maillard et al., 2019).

Diffusion-kurtosis imaging (DKI) is a higher order diffusion model that represents another evolution of the DTI, accounting for non-Gaussian diffusion of water molecules (Jensen et al., 2005; Jensen & Helper, 2010). Metrics derived from DKI, particularly mean kurtosis (MK), have been suggested as putative indices of glymphatic activity (Örzsik et al., 2023). In fact, it was recently shown that the expansion of interstitial space associated with sleep results in a reduction of diffusion non-Gaussianity and hence of diffusion kurtosis.

Besides MRI-based markers, recent data suggest a potential utility of quantifying CSF-AQP4 levels for the detection of glymphatic dysregulation (Bergström et al., 2021). AQP4 is a water channel densely expressed on the perivascular side of astrocytes lining PVS. Its polarized localization is essential for the rapid influx of fluid from periaxonal spaces into the brain parenchyma. In neurodegeneration due to AD pathology, AQP4 is known to be over-expressed and mislocalized away from astrocytic endfeet processes (Boespflug et al., 2018; Zeppenfeld et al., 2017), possibly impairing glymphatic clearance of waste solutes and pathological proteins (Buccellato et al., 2022; Lopes et al., 2024). Consistent with this, levels of AQP4 were found to be increased in the CSF of patients affected by neurodegenerative dementia, particularly AD (Arighi et al., 2022; Bergström et al., 2021). Moreover, preliminary data suggest an association between CSF levels of AQP4, tau protein levels (Bergström et al., 2021) and enlarged PVS burden, particularly in the CSO (Sacchi et al., 2023).

Different biomarkers may preferably mirror alterations in one the three compartments of the GS. Although the precise anatomical substrate of enlarged PVS is still under debate, the overall evidence suggests that they represent periaxonal spaces (Wardlaw et al., 2020). Given the close spatial and functional relationship between PVS and AQP4, the latter might primarily represent a marker of periaxonal pathophysiological mechanisms. Nonetheless, AQP4 is also expressed on brain capillaries and veins (Iliff et al., 2012), and thus might

potentially indicate more widespread aspects of GS dysfunction. FW and MK mainly mirror changes in parenchymal microstructure and fluid content, providing information about ISF, while the DTI-ALPS index is a measure of the diffusivity along the PVS around the deep medullary veins. Together, all these biomarkers may enable an indirect evaluation of the whole GS *in vivo*.

Against this background, the principal aim of this study was to evaluate the changes of these putative biomarkers of GS function in patients with neurodegenerative diseases, particularly AD, and with stable mild cognitive impairment (MCI) in respect to healthy elderly people. The secondary aims were to evaluate the reciprocal correlations among GS biomarkers and the correlations between these parameters and established markers of AD pathology and neurodegeneration and cognitive scores.

## 2 | MATERIALS AND METHODS

### 2.1 | Study participants

For this study, we prospectively enrolled a cohort of subjects from those referred to the Neurodegenerative Diseases Unit of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico in Milan (Italy) in suspicion of dementia, over a 12-month period. In addition to the assessments needed to formulate (or exclude) a specific diagnosis of dementia, recruited participants were required to undergo an additional 10-min long acquisition (i.e., multi-shell DW-MRI) at the end of their MRI clinical scan, and to give permission to use their clinical data (in anonymous form) for research purposes. Whenever a CSF examination was clinically appropriate, subjects were given the option to agree to the collection of an extra CSF sample for quantification of AQP4 levels.

At baseline, in all subjects the diagnostic work-up included past medical history; general and neurological examination; neuropsychological assessment; MRI scanning; and, when appropriate, CSF examination.

All recruited patients were clinically followed-up every 6 months for at least 1 year, and reclassified as suffering from a progressive neurodegenerative disease (AD or non-AD type), or a non-degenerative condition (stable MCI) if they were discharged with no evidence of neurodegeneration and did not show cognitive deterioration at follow-up. Cognitively unimpaired (CU) individuals presented with subjective memory complaints and underwent an extensive diagnostic work-up, which did not provide any evidence for cognitive impairment or an ongoing neurodegenerative condition.

Exclusion criteria for enrolment in the study were: (a) previous history of major psychiatric disorders; (b) MRI evidence of remarkable vascular pathology, cerebral amyloid angiopathy (CAA), or intracranial space-occupying lesions; (c) coarse movement artifacts on MRI scans. The study involving human participants was reviewed and approved by the Local Ethics Committee (Comitato Etico Area 2 Milano, approval N 859\_2021, date 14.09.2021). All participants and their legal guardians (when appropriate) were required to provide written informed consent before entering the study.

## 2.2 | MRI acquisition

MRI data were acquired on a Philips Achieva dStream 3 T scanner (Eindhoven, Netherlands) in a single scanning session. All the subjects underwent the same acquisition protocol, which included:

1. A volumetric high-resolution T1-weighted magnetization prepared gradient recalled echo images (MPRAGE; TE = 3.81 ms; TR = 8.27 ms; flip angle = 8°; matrix = 240 × 240 × 180; slice thickness = 1 mm);
2. A volumetric fast fluid-attenuated inversion recovery (FLAIR: TE = 300 ms; TR = 5000 ms; TI = 1700 ms; echo-train-length = 182, flip angle = 90°; matrix = 240 × 240 × 180; slice thickness = 1 mm; slice spacing = 1 mm)
3. A multi-shell DW-MRI scan (TE = 85 ms, TR = 8400,  $b$  values = 1000/2000  $\text{mm}^{-2}$ , number of diffusion directions = 32/32, FoV = 240 × 240  $\text{mm}^2$ , matrix = 96 × 96, number of slices = 60; slice thickness = 2.5 mm, phase encoding = PA, nine b0 images (PA), three additional b0 images (AP)).

Fifty five subjects were scanned in the morning, while the remaining half (56/111) in the afternoon.

## 2.3 | Regions of interest segmentation

T1-weighted images (T1w) were pre-processed and segmented using FreeSurfer 7.11 to obtain binary mask of WM and BG. To reduce the odds of PVS misclassification and partial volume effects, the binary WM mask underwent erosion by one voxel, followed by the subtraction of a dilated mask of the lateral ventricles.

## 2.4 | White matter lesion segmentation

White matter lesions (WML) were segmented applying the lesion growth algorithm (LGA) as implemented in the Lesion Segmentation Tool (LST) toolbox version 3.0.0 ([www.statistical-modelling.de/lst.html](http://www.statistical-modelling.de/lst.html)) from Statistical Parametric Mapping (SPM12, Wellcome Trust Centre for Neuroimaging), setting the initial  $k$  value to 0.2. The resulting probability map was then binarized using a threshold of 0.5.

Tissue probability maps of gray matter (GM), WM, and CSF generated in the first step of the LGA were also used for total intracranial volume (TIV) calculation. The WML (WMLVF;  $\text{WMLVF} = \text{WML volume}/\text{TIV}$ ) and GM (GMVF;  $\text{GMVF} = \text{GM volume}/\text{TIV}$ ) volume fractions were obtained to correct for interindividual brain size variability.

## 2.5 | PVS rating and segmentation

Enlarged PVS were assessed both visually and quantitatively. First, according to Paradise et al. (2020) the number of enlarged PVS in the slices located 2 mm (BG<sub>vis</sub>) and 37 mm (CSO<sub>vis</sub>) above the anterior commissure and their sum (ALL<sub>vis</sub>) were counted by LS, a neurologist

trained in PVS scoring. PVS were defined according to the Standards for Reporting Vascular Changes on Neuroimaging (STRIVE) criteria (Duering et al., 2023; Wardlaw et al., 2013). PVS of any diameter were included if they fulfilled other STRIVE diagnostic criteria.

Then, PVS were mapped from T1w images using an automated quantification method as follows. Native T1w images were denoised using the non-local mean algorithm available in the scikit-image library 0.21 (<https://scikit-image.org/>) (van der Walt et al., 2014), N4 bias corrected (Tustison et al., 2010) and skull-stripped. Then, Frangi filter (Frangi et al., 1998) was applied to preprocessed T1w images using the scikit-image library 0.21, and setting default parameters with a scale range of 0.5–5 voxels to maximize vessel inclusion. The resulting vesselness map was then binarized using a raw threshold of 0.05 for WM and 0.1 for BG, determined by consensus among LS, FD, and CC after visual inspection of the Frangi output. To eliminate PVS mis-segmentation caused by WM hyperintensity, WML were excluded from the PVS mask. The PVS volume fraction (PVSVF;  $\text{PVSVF} = \text{PVS volume}/\text{TIV}$ ) was measured in the WM and BG and then added to obtain the volume of all PVS (ALL).

## 2.6 | FW and MK calculation

DW images were first corrected for susceptibility induced distortions using the FSL TopUp function (FSL, version 6.0.1) and the additional b0 images, followed by eddy-current correction (Andersson & Sotiropoulos, 2016). To address motion-associated signal dropout, slice-wise outliers were estimated and replaced using Gaussian Process prediction (Andersson & Sotiropoulos, 2016).

Maps of the fractional volume of FW and of the MK were constructed from preprocessed DW images fitting a regularized bitensor model and the DKI, respectively, with the open-source software package Diffusion Imaging in Python (Dipy) algorithm ([dipy.org/](http://dipy.org/)) (Garyfallidis et al., 2014). A 3D Gaussian smoothing kernel (FWHM = 1.25 mm) was applied to the DW images before fitting the model.

Images were then co-registered to their corresponding high resolution T1w image using SPM12.

Mean white matter FW (FW-WM) and MK (MK-WM) values were measured within each WM mask, after excluding WML and PVS and reslicing the WM mask to match the resolution of the DW images, thus including the normal appearing WM only.

## 2.7 | DTI ALPS

The DTI-ALPS index was calculated from DW images using a semi-automated and highly reliable pipeline developed and validated by Taoka, Ito, Nakamichi, Kamagata, et al. (2022).

Fractional anisotropy (FA) and mean diffusivity (MD) maps as well as diffusivity maps along the directions of the x-axis (right–left; Dxx), y-axis (anterior–posterior; Dyy), and z-axis (inferior–superior; Dzz) were obtained from preprocessed DW images applying the DTI model

with Dipy. The FA maps from all participants were first linearly and then nonlinearly registered to the high-resolution FMRIB58\_FA standard space image. The subject with the smallest degree of warping was selected for the regions of interest (ROIs) placement. Using this subject's color-coded FA map, 5 mm spherical ROIs were placed in the projection and association areas at the level of the lateral ventricle bodies in the left and right hemispheres. ROIs positioning was visually checked in each participant, and manual corrections were performed where needed.

The ALPS index was calculated as reported by Taoka et al. (2017). Left hemisphere ALPS (ALPS\_left), right hemisphere ALPS (ALPS\_right) and their average (ALPS\_mean) were obtained. Average FA and MD of the normal appearing WM were also derived.

## 2.8 | CSF AQP4 and protein determination

Available CSF samples were centrifuged at  $1500 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatants were aliquoted in polypropylene tubes and stored at  $-80^{\circ}\text{C}$  until use. CSF levels of A $\beta$ 42, total tau (T-tau), and phosphorylated tau at 181 (P-tau) were assessed using either a ChemiLuminescence Enzyme ImmunoAssay (CLEIA) by a Lumipulse G600II platform (Fujirebio, Ghent, Belgium). The following normality thresholds were used:  $>640$  pg/mL for A $\beta$ 42,  $<61$  pg/mL for P-tau, and  $< 580$  pg/mL for T-tau (Sacchi et al., 2022).

AQP4 concentration was determined in all available samples of CSF using a specific ELISA kit from Cusabio ([www.cusabio.com](http://www.cusabio.com)), as detailed elsewhere (Arighi et al., 2022; Sacchi et al., 2023).

## 2.9 | Statistical analysis

Data were analyzed using R. 4.3.1. Considering that, except for MK-WM, all variables exhibited significant skewness, non-parametric testing was favored. The Kruskal-Wallis tests was employed to assess differences in demographic and clinical characteristics among all groups. Generalized linear models were utilized to investigate differences in GS biomarkers, employing a gamma distribution and a log link function, while incrementally entering additional covariates.

In the basic model (Model 1), age, sex, years of formal education and—for MRI indices only—scanning time (dichotomous) were included. Model 2 incorporated WMLVF and Model 3 WMLVF and GMVF as covariates to account for the influence of WM macrostructural damage and GM atrophy. Model 1 was employed to evaluate differences in all biomarkers; Model 2 and 3 were specifically applied when analyzing MRI parameters (i.e., all except AQP4). Two runs of analysis were conducted, the first one including all patients; the second one after dividing both patients with AD and patients with other neurodegenerative diseases into two subgroups, based on the degree of GM atrophy using the median value of GMVF in each subgroup. Statistical significance was set at an uncorrected  $p$ -value of  $<.05$ .

Finally, partial Spearman rank correlation tests, adjusted for age, sex and years of education were conducted to assess reciprocal

associations among putative GS biomarkers and their potential relationships with AD CSF biomarkers and Mini-Mental State Examination (MMSE) scores. The false discovery rate (FDR) was used to correct for multiple correlation tests.

## 3 | RESULTS

### 3.1 | Demographic and clinical characteristics of the study population

A total of 111 subjects were included in the study. Seventy one of them were diagnosed as suffering from a neurodegenerative disease according to the most recent diagnostic criteria available for each condition (Armstrong et al., 2013; Crutch et al., 2017; Dubois et al., 2021; Gorno-Tempini et al., 2011; McKeith et al., 2017; Rascovsky et al., 2011): Forty seven patients with AD while 11 with FTD, 6 with DLB and 7 with other neurodegenerative disease (non-AD [nAD] = 24). Based on their clinical follow-up, 17 subjects were diagnosed with stable MCI while 23 were classified as CU individuals.

Fifty out of 111 participants also underwent lumbar puncture for CSF analysis and 49/50 samples were available for AQP4 measurement (nAD = 12, AD = 29, MCI = 4, CU = 4).

Demographic and clinical characteristics of the study population are summarized in Table 1. Notably, MCI patients were less educated than AD patients ( $p = .02$ ). No differences in age, sex and disease duration were found between groups.

### 3.2 | Group comparisons in the whole cohort

AQP4 levels were significantly higher in AD ( $\exp(b) = 2.05$ , [Confidence Interval (CI) = 1.25–3.21],  $p = .005$ ) and nAD patients ( $\exp(b) = 2.98$ , CI = [1.76–4.85],  $p < .001$ ) compared to CU individuals. Patients with AD showed a higher FW-WM ( $\exp(b) = 1.06$ , CI = [1.00–1.11],  $p = .043$ ) than CU individuals (Figure 1).

In Models 2 and 3, AD patients still showed significantly higher FW-WM than CU subjects ( $\exp(b) = 1.06$ , CI = [1.01–1.12],  $p = .028$  in Model 2;  $\exp(b) = 1.06$ , CI = [1.01–1.12],  $p = .031$  in Model 3) (Figure 4a).

### 3.3 | Group comparisons including only degenerative patients with less GM atrophy

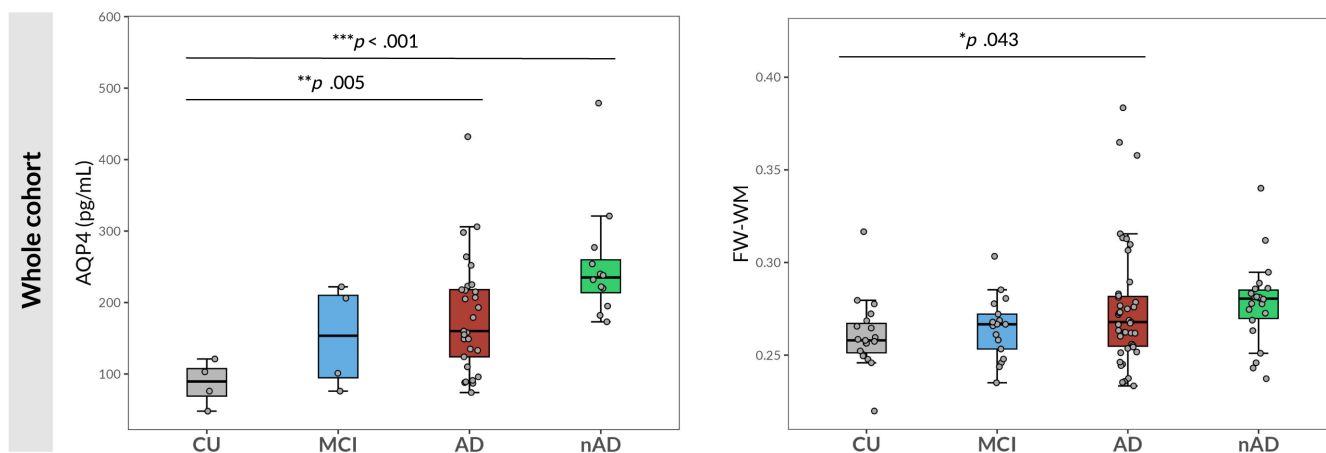
When considering AD ( $n = 23$ ) and nAD ( $n = 12$ ) patients with less GM atrophy, AQP4 and FW-WM were significantly higher in the AD ( $\exp(b) = 2.20$ , CI = [1.29–3.59],  $p = .006$ ;  $\exp(b) = 1.08$ , CI = [1.01–1.15],  $p = .019$ , respectively) and in the nAD groups ( $\exp(b) = 2.66$ , CI = [1.50–4.62],  $p = .002$ ;  $\exp(b) = 1.10$ , CI = [1.02–1.19],  $p = .019$ , respectively) compared to CU subjects (Figure 2). In Models 2 and 3, FW-WM was still higher in both AD ( $\exp(b) = 1.08$ , CI = [1.01–1.15],  $p = .023$ , Model 2;  $\exp(b) = 1.08$ , CI = [1.01–1.15],  $p = .029$ ,

**TABLE 1** Demographics and clinical characteristics of the study population.

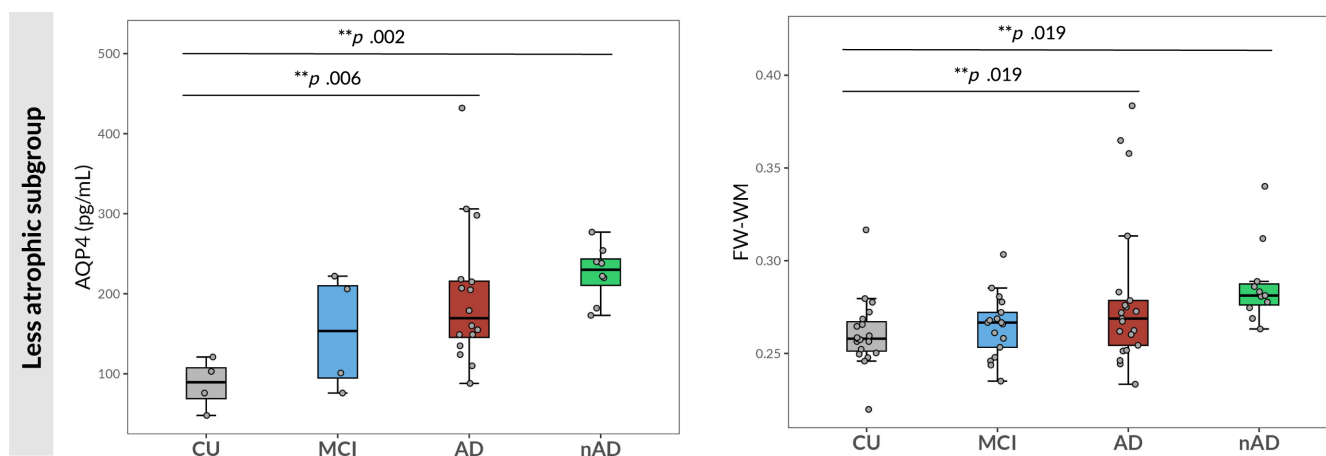
	nAD	AD	MCI	CU	p-Value (fdr)						
	n = 24	n = 47	n = 17	n = 23	nADvsADvs MCIvsCU	nADvs AD	nADvs MCI	ADvs MCI	nADvs CU	ADvs CU	MCIvs CU
Sex (M/F)	9/15	22/25	8/9	14/9	.36						
Age (y)	75 (70.75, 77.25)	71 (67.5, 76)	74 (69, 76)	70 (60, 75.5)	.12						
Education (y)	13 (7.75, 14.25)	13 (12, 18)	8 (8, 13)	13 (8, 18)	<b>.042</b>			<b>.031</b>			
Disease duration (y)	3.02 (2.32, 5.6)	2.51 (1.69, 4.69)	2.73 (1.70, 3.48)	2.80 (2.30, 4.22)	.49						
MMSE (N)	24 (18.75, 26)	23 (20.5, 26)	27 (25, 28)	29 (28, 30)	<b>&lt;.001</b>		<b>.012</b>	<b>.001</b>	<b>&lt;.001</b>	<b>&lt;.001</b>	<b>.001</b>
A $\beta$ 42 (pg/mL)	829 (700, 891)	496 (435, 591)	1218 (1158.5, 1330)	854 (762, 945)	<b>&lt;.001</b>	<b>&lt;.001</b>	<b>.038</b>	<b>.001</b>			<b>.002</b>
T-tau (pg/mL)	518 (192, 851)	669 (407, 922)	252 (205, 261)	501 (276, 750)	0.056						
P-tau (pg/mL)	84 (33, 119)	122 (68, 154)	34 (30, 35)	88 (34, 140)	<b>.049</b>						
T-tau/A $\beta$ 42	0.78 (0.24, 1.11)	1.29 (0.78, 1.93)	0.19 (0.16, 0.21)	0.53 (0.38, 0.75)	<b>&lt;.001</b>	<b>.017</b>	<b>.038</b>	<b>.002</b>			<b>.031</b>
P-tau/A $\beta$ 42	0.12 (0.03, 0.17)	0.22 (0.15, 0.32)	0.02 (0.02, 0.03)	0.09 (0.05, 0.14)	<b>&lt;.001</b>	<b>.007</b>	<b>.046</b>	<b>.002</b>			<b>.046</b>
WMLVF	0.004 (0.002, 0.008)	0.003 (0.002, 0.007)	0.003 (0.001, 0.008)	0.002 (0.0006, 0.004)	0.1						
GMVF	0.371 (0.344, 0.425)	0.387 (0.354, 0.425)	0.418 (0.379, 0.454)	0.421 (0.398, 0.457)	<b>.013</b>		<b>.03</b>	<b>.03</b>			

Note: Data are presented as median (Q1, Q3). N represents the number of patients. p values reported in bold denote statistical significance at  $p < .05$  (Kruskal-Wallis test, FDR corrected).

Abbreviations: A $\beta$ 42, amyloid  $\beta$  42; AD, Alzheimer's disease patients; CU, cognitively unimpaired subjects; GMVF, gray matter volume fraction; MCI, mild cognitive impairment patients; MMSE, Mini-Mental State Examination; nAD, non Alzheimer's disease degenerative patients; P-tau, phosphorylated tau; T-tau, total tau; WMLVF, white matter lesion volume fraction.



**FIGURE 1** Group comparisons in the whole cohort. Boxplots of the differences in AQP4 levels and FW-WM among cognitively unimpaired (CU) individuals, patients with MCI, patients with AD and patients with degenerative diseases different from AD (nAD). The  $p$  values correspond to the generalized linear model analysis (Model 1). Statistical significance was set at  $p < .05$ .



**FIGURE 2** Group including only less atrophic degenerative patients. Boxplots of the differences in AQP4 levels and FW-WM among cognitively unimpaired (CU) individuals, patients with MCI, patients with AD with lower gray matter atrophy and patients with degenerative diseases different from AD and lower gray matter atrophy (nAD). The  $p$  values correspond to the generalized linear model analysis (Model 1). Statistical significance was set at  $p < .05$ .

Model 3) and nAD groups ( $\text{exp}(b) = 1.10$ ,  $\text{CI} = [1.02-1.19]$ ,  $p = .021$ , Model 2;  $\text{exp}(b) = 1.10$ ,  $\text{CI} = [1.02-1.19]$ ,  $p = .023$ , Model 3) compared to CU individuals (Figure 4b).

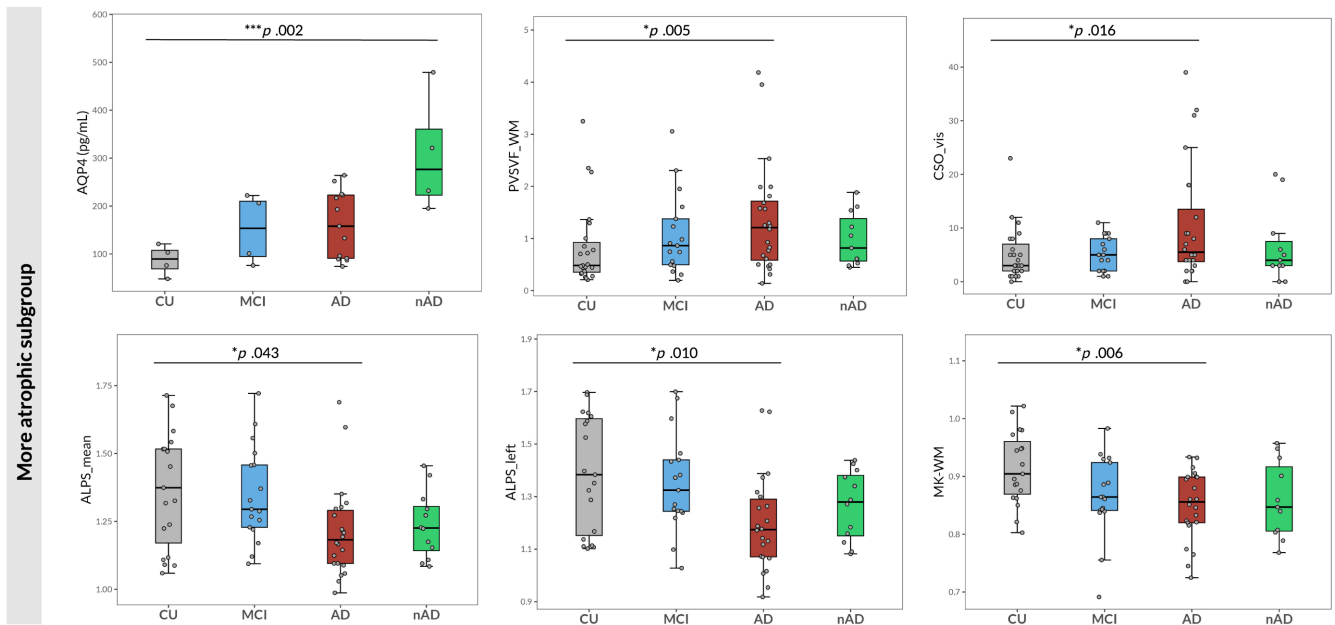
### 3.4 | Group comparisons including only degenerative patients with higher GM atrophy

When focusing on AD ( $n = 24$ ) and nAD ( $n = 12$ ) patients with more remarkable GM atrophy, the former group showed higher CSO\_vis ( $\text{exp}(b) = 1.89$ ,  $\text{CI} = [1.15-3.10]$ ,  $p = .016$ ), ALL\_vis ( $\text{exp}(b) = 1.59$ ,  $\text{CI} = [1.12-2.24]$ ,  $p = .013$ ), PVSVF\_WM ( $\text{exp}(b) = 1.89$ ,  $\text{CI} = [1.23-2.90]$ ,  $p = 0.005$ ) and PVSVF\_ALL ( $\text{exp}(b) = 1.50$ ,  $\text{CI} = [1.04-2.16]$ ,  $p = .036$ ) together with lower MK-WM ( $\text{exp}(b) = 0.94$ ,  $\text{CI} = [0.90-0.98]$ ,  $p = .006$ ), ALPS\_mean ( $\text{exp}(b) = 0.91$ ,  $\text{CI} = [0.84-0.99]$ ,

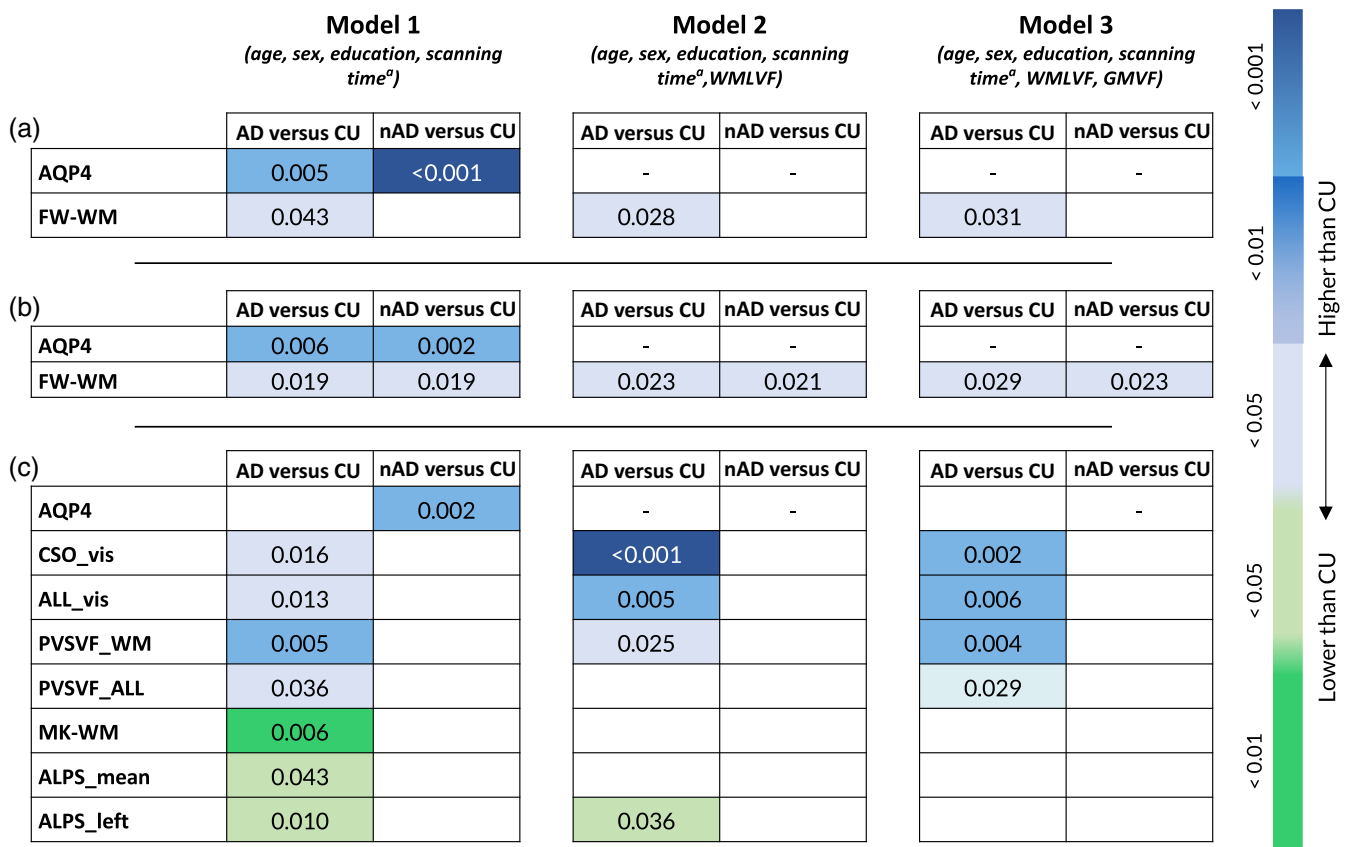
$p = .043$ ) and ALPS\_left ( $\text{exp}(b) = 0.88$ ,  $\text{CI} = [0.81-0.97]$ ,  $p = .010$ ). Moreover, nAD patients showed higher levels of AQP4 ( $\text{exp}(b) = 3.39$ ,  $\text{CI} = [1.76-6.49]$ ,  $p = .002$ ) compared to CU individuals, while, in the same comparison, AD patients showed a trend toward statistical significance only ( $p = .052$ ) (Figure 3).

In Model 2, CSO\_vis ( $\text{exp}(b) = 2.42$ ,  $\text{CI} = [1.47-3.97]$ ,  $p < .001$ ), ALL\_vis ( $\text{exp}(b) = 1.72$ ,  $\text{CI} = [1.21-2.46]$ ,  $p = .005$ ) and PVSVF\_WM ( $\text{exp}(b) = 1.64$ ,  $\text{CI} = [1.08-2.48]$ ,  $p = .025$ ) remained significantly higher, whereas ALPS\_left significantly lower ( $\text{exp}(b) = 0.90$ ,  $\text{CI} = [0.82-0.99]$ ,  $p = .036$ ) in AD patients compared to CU subjects. Conversely, differences in MK-WM, PVSVF\_ALL and ALPS\_mean did no longer reach statistical significance.

In Model 3, results obtained in Model 2 were partially preserved. AD patients still showed higher CSO\_vis ( $\text{exp}(b) = 2.54$ ,  $\text{CI} = [1.39-4.58]$ ,  $p = .002$ ), ALL\_vis ( $\text{exp}(b) = 1.83$ ,  $\text{CI} = [1.19-2.79]$ ,  $p = .006$ ),



**FIGURE 3** Group comparisons including only more atrophic degenerative patients. Boxplots of the differences in AQP4 levels, PVSVF\_WM, visual scores of PVS in the CSO, ALPS\_mean, ALPS\_left and MK-WM among cognitively unimpaired (CU) individuals, patients with MCI, patients with AD with higher GM atrophy and patients with degenerative diseases different from AD with higher GM atrophy (nAD). The p values correspond to the generalized linear model analysis (Model 1). Statistical significance was set at  $p < .05$ .



**FIGURE 4** Heatmaps summarizing significant differences in GS biomarkers among groups. <sup>a</sup>for MRI biomarkers only. (a) comparisons in the whole cohort; (b) comparisons including AD and nAD patients with lower GM atrophy; (c) comparisons including AD and nAD patients with higher GM atrophy.



PVSVF\_WM ( $\exp(b) = 2.07$ ,  $CI = [1.27-3.32]$ ,  $p = .004$ ) and PVSVF\_ALL ( $\exp(b) = 1.59$ ,  $CI[1.05-2.41]$ ,  $p = .029$ ) than CU. However, the observed difference in ALPS\_left was no longer significant (Figure 4c).

### 3.5 | Correlation analysis in the whole population

Considering the entire study population, CSO\_vis exhibited positive association with increased FW-WM ( $p_{\text{fdr}} < .001$ ). MK-WM was positively correlated with ALPS\_right and ALPS\_mean ( $\rho = 0.29$ ,  $p_{\text{fdr}} = .019$ ;  $\rho = 0.27$ ,  $p_{\text{fdr}} = .046$ , respectively). GS MRI parameters were not associated with CSF biomarkers of AD nor MMSE in the whole population.

### 3.6 | Correlation analysis in the AD subgroup

When focusing on patients with AD, higher PVSVF\_WM, PVSVF\_ALL, and CSO\_vis were associated with higher FW-WM ( $\rho = 0.46$ ,  $p_{\text{fdr}} = .018$ ;  $\rho = 0.44$ ,  $p_{\text{fdr}} = .028$ ;  $\rho = 0.52$ ,  $p_{\text{fdr}} = .006$  respectively). PVSVF\_WM and PVSVF\_ALL showed a significant negative correlation with ALPS\_right ( $\rho = -0.48$ ,  $p_{\text{fdr}} = .015$ ;  $\rho = -0.37$ ,  $p_{\text{fdr}} = .027$  respectively).

After correction for multiple correlations, lower ALPS\_right, ALPS\_left, and ALPS\_mean showed a trend toward association with lower A $\beta$ 42 levels ( $\rho = 0.57$ ,  $p_{\text{fdr}} = .095$ ;  $\rho = 0.52$ ,  $p_{\text{fdr}} = .095$ ;  $\rho = 0.53$ ,  $p_{\text{fdr}} = .095$  respectively). GS MRI parameters were not associated with MMSE in the AD subgroup.

Uncorrected correlation between GS biomarkers and FA, MD, WMLVF, GMVF, and age are reported in Supplementary Table 1.

## 4 | DISCUSSION

In this study, we combined the use of non-invasive MRI metrics and measurement of CSF-AQP4 levels to attempt a comprehensive characterization of the GS function in patients suffering from neurodegenerative dementia, with a specific focus on AD.

According to our a priori hypothesis, we observed significant alterations of all the examined biomarkers in patients with AD and, to a lesser extent, in those with other forms of degenerative dementia when compared to CU individuals. In particular, patients with degenerative diseases exhibited higher CSF-AQP4 levels and increased FW-WM compared to CU subjects. Moreover, AD patients compared to CU subjects showed a greater burden of enlarged PVS in the WM, reduced MK and diffusivity along perivascular spaces obtained by ALPS index, though these latter findings were statistically significant only when focusing the analysis on AD patients with higher GM atrophy.

Overall, these findings suggest an alteration of the GS in association with neurodegeneration and AD pathology. However, variations among different patient subgroups and the absence of correlation with cognitive scores require further discussion.

Previous investigations have consistently demonstrated an association between worsening AD pathology and over-expression and loss of perivascular localization of AQP4 (Boespflug et al., 2018; Zeppenfeld et al., 2017). In line with this, ourselves and other groups have previously described higher levels of AQP4 in the CSF of patients with AD and other forms of degenerative dementia, when compared to healthy controls (Arighi et al., 2022). Here, we replicated the same finding in an entirely new cohort of patients, which indicates robustness of this observation and its potential pathophysiological value. However, AQP4 levels were not correlated with tau, contrary to previous observations from our group (Arighi et al., 2022; Sacchi et al., 2023), and were unrelated to the other biomarkers of glymphatic activity, hindering speculation regarding the pathophysiological origin of AQP4 over-expression.

Further investigation would help to elucidate whether AQP4 may be considered either a proxy measure of glymphatic dysfunction or rather a marker of reactive astrocytosis in the context of neurodegeneration.

The significantly higher FW in the normal appearing WM of patients affected by AD and other neurodegenerative diseases is consistent with previous data (Bergamino et al., 2021; Dumont et al., 2019; Ji et al., 2017; Kamagata et al., 2022). Indeed, two studies analyzing ADNI data (Dumont et al., 2019; Kamagata et al., 2022), along with a third monocentric study from Singapore (Ji et al., 2017), consistently reported higher FW in the normal appearing WM of AD patients compared to controls. Conversely, we did not observe a significant difference in FW-WM between MCI patients and CU subjects, aligning with findings by Kamagata et al. (2022), but contrasting with those by Dumont et al. (2019). In our study, increase of FW content was particularly pronounced within the subgroup of neurodegenerative patients exhibiting a lower degree of GM atrophy. Moreover, it was only marginally affected when accounting for WMLVF, in contrast to what has been observed by Kamagata et al. (2022) and GMVF.

Taken altogether, these observations indicate that FW might reflect early interstitial water accumulation, which is at least partially independent from WM damage and GM atrophy. This phenomenon might therefore signify expansion and stasis of ISF (Chad et al., 2018), possibly due to glymphatic dysfunction. However, several non-mutually exclusive mechanisms, including edema, modulation of BBB permeability, and neuroinflammation (Pasternak et al., 2012) are known to occur and may result in an increase in extracellular water fraction. The observed association between increase FW-WM and a higher burden of enlarged PVS in the WM may support an interpretation of this metric as a marker of impaired interstitial bulk flow.

Differences in the other biomarkers were only detected in AD patients with a higher degree of GM atrophy compared to CU individuals. This result may prompt some general considerations. First, regarding brain atrophy as a surrogate of disease severity, AQP4, and FW might capture early alterations in glymphatic function, possibly linked to an astrocytic response to damage (Iliff et al., 2014; Ren et al., 2013). In contrast, the remaining biomarkers might reflect later pathophysiological aspects of glymphatic decompensation. This for

example, has been suggested for ALPS-index (Ma et al., 2021). Second, although glymphatic failure is thought to be a common mechanism in neurodegenerative diseases, the distinctive pattern of alterations that we observed in the AD subgroup may imply a specific pathophysiological role of GS dysfunction in AD brains. This is particularly evident in the case of ALPS index and PVS, whose deviations from normal levels were more pronounced in AD patients compared to those with other neurodegenerative dementias. Additional considerations pertaining to individual biomarkers are discussed in the following sections.

When comparing patients with AD and more pronounced GM atrophy against CU subjects, the former showed PVS enlargements more significantly distributed in the WM but not in the BG. Post-mortem studies have reported direct correlations between PVS in the WM, cortical A $\beta$  deposition, CAA severity, and APOE  $\epsilon$ 4 polymorphism (Perosa et al., 2022; Roher et al., 2003; van Veluw et al., 2016). Nonetheless, some studies failed to identify an association between PVS in the WM and amyloid load, as assessed by either positron emission tomography (Banerjee et al., 2017; Wang et al., 2021) or CSF-A $\beta$  levels (Maier-Hein et al., 2015; Murdock et al., 2024). A possible explanation for this inconsistency is that enlarged PVS in the WM are associated with AD but not A $\beta$  biomarkers because they are manifestations amyloid-independent processes of AD, for example a tau protein-related process. In fact, ourselves and other groups have previously described a significant association between tau levels in the CSF (Chad et al., 2018; Maier-Hein et al., 2015) or tau deposition (Wang et al., 2021) and enlarged PVS, particularly in the CSO (Moses et al., 2022). Indeed, differences in PVS burden in the WM of AD patients were enhanced by the inclusion of WMLVF and GMVF as confounding factors. This suggests that enlarged PVS burden in AD exceeds what is associated with vascular pathology and could be linked to accelerated neurodegeneration secondary to GS dysfunction.

DKI derived metrics were recently identified as a potential biomarker of glymphatic clearance (Örzsik et al., 2023). According to available data, we hypothesized a reduction in MK of the normal appearing WM associated with ISF expansion secondary to GS dysfunction. We observed the expected changes when comparing patients with higher levels of atrophy against CU individuals. This was not the case when comparing the whole cohort of patients or the less atrophic subgroup to CU subjects. Considering GM atrophy as a proxy of disease stage, these findings may be partially explained by the non-linear relationship between amyloid burden and changes in WM diffusivity (Benitez et al., 2022; Collij et al., 2021) across disease progression. We argue that the less remarkable changes in WM diffusivity seen at early AD stages may reflect a mixture of compensating and inflammatory mechanisms, which are followed by more widespread changes in diffusivity driven by neurodegeneration. Moreover, MK values were strongly dependent on GMVF, aging and measures of WM integrity (Supplementary Table 1). This suggests that MK reduction may primarily reflect myelin sheath damage and decreased axonal density. In contrast to other techniques (e.g., FW), MK is likely unable to single out the increase in interstitial volume fraction due to GS dysfunction and ISF stasis in the context of neurodegeneration.

Overall, previous studies reported a significant decrease in ALPS index of AD patients, and less consistently MCI (Steward et al., 2021; Zhong et al., 2023), as compared to controls (Kamagata et al., 2022; Steward et al., 2021; Taoka et al., 2017; Zhang, Wang, et al., 2023; Zhong et al., 2023). Our findings are partially consistent with these data. We observed a significant reduction of ALPS index in AD patients with more GM atrophy, but not in the less atrophic subgroup. Moreover, we did not detect any significant difference between patients with MCI and CU, in contrast to results by Steward et al. (2021) and Zhong et al. (2023) but as already described by Kamagata et al. (2022). The inconsistent findings in relation to MCI patients may be due to the lack of consensus about the clinical diagnostic criteria and their operationalization in different studies, leading to prominent heterogeneity of the MCI groups across different studies. Indeed, a recent study by Huang (2024) found significantly lower ALPS index values in amyloid-positive, but not amyloid negative MCI patients compared to controls. Considering the defining features of our MCI group, this result may help explain our finding. Conversely, the absence of significant differences in ALPS-indices when incorporating less atrophic patients need to be further addressed. Most of our AD patients could be classified as mild. By contrast, previous studies have mostly included AD patients at more advanced disease stages (Steward et al., 2021; Taoka et al., 2017; Zhang, Wang, et al., 2023; Zhong et al., 2023), which could partially account for some of the inconsistencies. Interestingly, in a recent study by Kamagata et al., a significant reduction of mean ALPS index was detectable even in patients with rather mild AD who were selected from the ADNI database. Given the comparable sample size and the similar statistics, such a discrepancy might be attributable to their incorporation of APOE  $\epsilon$ 4 as a confounding factor. Unfortunately, we do not have APOE  $\epsilon$ 4 data available for our cohort of patients to further support this speculation.

Moreover, as already pointed out by Kamagata et al., the differences in ALPS indices were significantly reduced after the inclusion of WMLVF as covariate, and disappeared after the additional incorporation of GMVF in the models, indicating the influence of WML and GM atrophy on measures of glymphatic dysfunction in AD brains (Hsu et al., 2023; Naganawa et al., 2019; Siow et al., 2022; Song et al., 2022; Taoka, Ito, Nakamichi, Nakane, et al., 2022). However, based on their own findings, Hsu et al. have recently proposed that the decline in the ALPS index may precede and partially mediate GMVF alterations in individuals with AD (Hsu et al., 2023). Furthermore, Huang et al. observed that the abnormality of ALPS index prominently increased before the threshold of CSF A $\beta$ 42 positivity and then plateaued (Huang, 2024). Nevertheless, in the absence of any conclusive evidence from longitudinal data or animal models (Harrison et al., 2020; Iliff et al., 2012; Peng et al., 2016), an alternative interpretation is that changes in ALPS index might represent a late epiphenomenon of structural brain damage.

The strong association between ALPS index, WM damage and GM atrophy may also explain its association with patient cognitive scores, particularly with the MMSE of more atrophic patients (Hsu et al., 2023; Taoka et al., 2017; Zhang, Wang, et al., 2023; Zhong et al., 2023). We did not detect any association in the whole cohort.

This is contrary to findings in most prior studies but aligns with recent research by Matsushita et al. (2023). In both cases, disagreement may be attributable to patient stratification (predominantly very mild to mild AD) and the relatively narrow range of MMSE scores.

As previously described (Kamagata et al., 2022), we also observed a trend toward association between ALPS index in the left hemisphere and CSF-A $\beta$ 42 in the AD subgroup. It is notable that the observed trend was specific to the left ALPS. This could be attributed to anatomical differences between the two hemispheres and the inclusion of patients affected by progressive aphasia, which primarily impacts the left hemisphere. Nonetheless, the relationship between ALPS index and amyloid deposition in the cerebral cortex remains unclear (Hsu et al., 2023; Matsushita et al., 2023) and requires further investigations.

In summary, according to our study, ALPS index may behave like a proxy measure of late GS failure associated with neurodegeneration, rather than an early biomarker of glymphatic dysfunction. On the other hand, the distinct changes observed in ALPS within the AD subgroup and its correlation with A $\beta$  suggest a specific association with accumulation of AD pathology.

## 5 | LIMITATIONS

The major limitation of this study is the lack of direct pathophysiological support to our measures. At the time of writing, only the DTI-ALPS index had been directly correlated with measures obtained with GBCA CSF tracers (Zhang et al., 2021). However, concerns still remain about its true capacity to represent glymphatic clearance, primarily based on anatomical considerations (Ringstad, 2024). Since the exact histopathologic processes underlying the observed alterations is not fully understood, our results need to be interpreted with caution.

Second, we only evaluated cross-sectional data from a single memory clinic. Future studies should focus on the longitudinal changes of these biomarkers and their interactions with amyloid and tau protein deposition and measure of cognitive dysfunction.

Third, the subgroups of MCI and CU individuals with available AQP4 dosing were rather small, because we prioritized homogeneity of the sample. However, statistical significances did not change when (1) joining MCI and CU groups AND (2) adding all CU patients with available AQP4 measurements from our lab (8 additional subjects) (Supplementary Figure 1).

Fourth, our PVS segmentation relied solely on T1w images due to the unavailability of T2 volumetric scans, in contrast to what has been suggested by Sepehrband et al. to enhance PVS contrast (Sepehrband et al., 2019). We also acknowledge the limitations of the PVS segmentation method. To deal with this limitation and strengthen our findings, we associated visual rating scores to volumetric PVS quantification.

Another limitation is that AQP4 genetic and APOE  $\epsilon$ 4 allelic variations were not included as confounding factors due to unavailability. AQP4 mutations are known to modulate glymphatic activity and associate with A $\beta$  clearance (Rainey-Smith et al., 2018; Sapkota

et al., 2022), while APOE allelic mutations may impact the number of PVS (Paradise et al., 2021; Shams et al., 2017). Furthermore, despite the exclusion of subjects with significant vascular brain damage, we did not control for cardiovascular risk factors and sleep disturbances, which are both known to influence glymphatic activity.

Lastly, we used GM atrophy to identify patients at different stages of the disease. Though evidence for this choice is sound (Alzheimer's disease Neuroimaging Initiative1, 2011; Jack et al., 2013; Planche et al., 2022), cognitive scores or severity scale could have been used instead. However, CDR was not available for all the participants, while the MMSE sensitivity is lower in the early stages of the disease due to cognitive reserve (Pettigrew et al., 2023; Zhu et al., 2021) and ceiling effect (Franco-Marina et al., 2010; Scollard et al., 2023). Moreover, it could not be representative of the disease stage in patients affected by subtypes of progressive aphasia, who were present, though rare, in both the nAD and the AD groups in our study.

## 6 | CONCLUSIONS

Our results revealed that AQP4 and FW-WM are significantly increased in patients with neurodegenerative dementia compared to CU subjects at the early stages of the disease as assessed by lower levels of GM atrophy. Additionally, we confirmed substantial GS alterations in more advanced AD patients, characterized by an increased burden of enlarged PVS in the WM, lower MK and lower DTI-ALPS. DTI-ALPS may also serve as a marker of disease progression in AD.

While the bulk of these findings align with existing literature reporting significant alterations of putative GS biomarkers in neurodegenerative diseases, particularly AD, we hereby describe a heavy dependency of some of these indices, particularly MK-WM and DTI-ALPS, on measures of structural brain damage. This may partly hinder their usefulness as standalone and indirect markers of glymphatic activity until validated by comparative studies against established gold standard.

Notwithstanding this limitation, our results suggest a close interaction between glymphatic function and neurodegeneration, particularly in the case of AD. This interaction could potentially serve as a target for dementia prevention, by managing physiological factors like sleep hygiene (Himali et al., 2023) and cardiovascular risk (Xie et al., 2024), which are known to influence GS activity. In addition, in patients who are already suffering from cognitive decline, this might represent a potential target for therapeutic interventions, whether pharmacological (Mohaupt et al., 2023) or physical (Murdock et al., 2024), capable of modulating glymphatic clearance.

## AUTHOR CONTRIBUTIONS

LS and AA designed the study, analyzed, and interpreted the data. LS drafted the manuscript. AA, TC, AMP, LG, FD, CC, and MP contributed to the analysis and interpretation of the data. MA, MS, and CF performed CSF analyses. MB, MC, BO, VP, FD, and CC helped in the analysis of MRI data. FT and GC acquired MRI data. AA, MB, LL, and

DG drafted and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

## INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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