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TESI DI LAUREA

Alteration of the glymphatic system in Alzheimer disease compared to non-Alzheimer dementia: a single center PET/MRI study

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ABSTRACT

Background: The glymphatic system clears brain waste via cerebrospinal fluid (CSF) perivascular routes, maintaining homeostasis, but its function is disrupted in neurological disorders such as Alzheimer disease (AD). The diffusion Tensor Imaging along the perivascular spaces (DTI-ALPS, Taoka *et al.* 2017) index serves as a non-invasive proxy for glymphatic functioning.

Aim: This study investigates the association between DTI-ALPS index and structural network alterations in people with dementia depending on amyloid positivity.

Methods: 104 subjects underwent brain DWI and amyloid PET scans in a PET/MR integrated system (3T, Siemens Biograph). Eighty-six of them had complete MRI data of enough quality. Of these, one patient was excluded for a diagnosis prone of interpretative bias. The resulting 85 patients were selected for further analyses. Subjects were divided into two groups based on qualitatively evaluated amyloid positivity on PET: 42 AMY+ (sex: 16F, age: 68.16 ± 7.07 years) and 43 AMY- (sex: 15 F, age: 67 ± 9 years). The AMY+ group was represented by AD (57%), PD/PDD/DLB (38%) and CAA (5%). The AMY- group was represented by patients with non-AD-related neurodegenerative diseases and was more heterogeneous. Diffusivity was evaluated at the level of the lateral ventricle body in spherical ROIs placed in projection and associative fibers to obtain the DTI-ALPS index. The ROIs were warped from standard space to DWI space passing through the high-resolution T1w. Whole-brain tractograms were generated through anatomically constrained probabilistic tractography as implemented in MRtrix3. We evaluated information transfer across the structural connectome using the characteristic path length (CPL) index.

Results: The AMY+ group exhibited a lower DTI-ALPS index as compared to AMY- group ($t = +2{,}78$, $p = 0.005$). The DTI-ALPS index did not correlate with disease severity and showed a direct correlation with age (younger aged had lower DTI-ALPS index). The CPL was significantly higher in the AMY+ group as compared to the AMY- ($t = -2.71$, $p = 0.006$). DTI-ALPS and CPL showed a significant negative association (Spearman's $\rho = -0.37$, $p = 0.023$).

Conclusions: The glymphatic system is impaired in people with cognitive decline and positive amyloid PET compared to people with non-AD cognitive decline. The degree of glymphatic dysfunction is associated with impaired global information transfer along the structural connectome.

RIASSUNTO

Background: Il sistema glinfatico depura il cervello dalle sostanze di scarto attraverso una complessa rete di canali perivascolari, contribuendo al mantenimento dell'omeostasi. Il suo funzionamento risulta alterato in diverse patologie neurologiche, tra cui la demenza di Alzheimer (AD). L'indice della diffusività a livello degli spazi perivascolari (DTI-ALPS, Taoka *et al.* 2017) costituisce un metodo non invasivo per valutare l'attività glinfatica.

Obiettivi: Lo scopo di questo studio è indagare l'associazione tra l'indice DTI-ALPS e le alterazioni del network cerebrale in soggetti con demenza, in relazione alla positività alla PET per amiloide.

Metodi: 104 soggetti sono stati sottoposti a risonanza magnetica in diffusione e PET per amiloide, tramite un sistema integrato PET/MR. (3T, Siemens Biograph). Dell'intero campione, 86 pazienti presentavano un imaging di qualità sufficiente; un paziente è stato escluso per una diagnosi soggetta a bias interpretativi. Gli 85 pazienti rimanenti sono stati inclusi nelle analisi successive. I soggetti sono stati suddivisi in due gruppi in base alla positività alla PET per amiloide: 42 AMY+ (sesso: 16 F, età: 68.16 ± 7.07 anni) e 43 AMY- (sesso: 15 F, età: 67 ± 9 anni). Il gruppo AMY+ comprendeva pazienti AD (57%), PD/PDD/DLB (38%) e CAA (5%) mentre il gruppo AMY- presentava una distribuzione diagnostica più eterogenea. La diffusività è stata analizzata a livello del corpo del ventricolo laterale in regioni di interesse sferiche (ROIs) posizionate a livello delle fibre di proiezione e di associazione, per calcoalre l'indice DTI-ALPS. Le ROIs sono state deformate dallo spazio standard allo spazio DWI passando attraverso immagini ad alta risoluzione pesate in T1. I trattogrammi dell'intero cervello sono stati generati tramite trattografia probabilistica anatomicamente vincolata (MRtrix3). Abbiamo valutato il trasferimento di informazioni attraverso il connettoma strutturale utilizzando l'indice di lunghezza del percorso caratteristico (CPL).

Risultati: il gruppo AMY+ ha mostrato un indice DTI-ALPS significativamente inferiore rispetto al gruppo AMY- ($t = +2{,}78$, $p = 0.005$). L'indice DTI-ALPS non è risultato correlato alla gravità della malattia mentre ha mostrato una correlazione diretta con l'età (i soggetti più giovani presentavano valori ALPS inferiori). L'indice

CPL era significativamente più alto nel gruppo AMY+ rispetto al gruppo AMY- (t = -2.71, p = 0.006). Inoltre, DTI-ALPS e CPL hanno mostrato una significativa correlazione negativa (ρ di Spearman = –0,37; p = 0,023).

Conclusioni: il sistema glinfatico risulta compromesso nei pazienti con declino cognitivo e positività alla PET per amiloide, rispetto a quelli con declino cognitivo non-AD. Il grado di disfunzione glinfatica è associato a una compromissione del trasferimento globale di informazioni lungo il connettoma strutturale.

INTRODUCTION

1. THE GLYMPHATIC SYSTEM

"It is necessary for men to know that from the brain come sensations of pleasure, joy, laughter, and play, as well as sorrow, distress, crying, and mourning. It is for this reason that one becomes mad, or exhibits emotions, or behaves badly when not in control. […] When the brain is healthy, everything works correctly. But if it becomes disturbed, inflamed, or dries up, it is necessary that one thinks poorly, becomes melancholic, mad, suffers from fears and terror both by night and by day, loses reasoning, becomes despondent and forgets many things and all of this happens against nature. When the brain is at rest, reasoning becomes clear and bright." [1]

In *On the sacred disease* Hippocrates extends his theory of the humors to the brain's health and disease. His general idea was that an imbalance of fluids could lead to the development of brain diseases, including cognitive decline. Even if modern medicine has widely surpassed this theory, the idea of a disrupted fluid balance and its connection with cognitive impairment is not too far from the modern concept of the glymphatic system and its involvement in the pathogenesis of neurodegenerative diseases such as Alzheimer disease (AD).

1.1 Definition and historical background

The glymphatic system is a recently described network of perivascular channels diving deep into the brain that allows a continuous exchange between the cerebral spinal fluid (CSF) and the interstitial fluid (ISF), thus playing an essential role for the clearance of waste substances. [2]

Fluid in excess and waste interstitial solutes are generally collected by the lymphatic system. [2] It is commonly known that the central nervous system (CNS) lacks conventional lymphatic vessels, which represents a paradox, given that lymphatic vessels are normally massively present in tissues with a high metabolic activity, such as the brain. [3] The perivascular channels, which have been recently identified in the brain as essential components of the glymphatic framework, have functional similarities to lymphatics. [4] Furthermore, the term "g-lymphatic" itself underlines a greater complexity than those of the peripheral lymphatic system, emphasizing the pivotal role of glial cells, whose vascular end feet define the

perivascular spaces (PVS). [4,5]. By addressing the CNS as an immune privileged system, meaning that it does not possess typically defined lymphatic vessels , the problem of how immune surveillance and waste clearance are conducted in its parenchyma promptly arises. [5] The identification of a glial-mediated ISF bulk flow and its interaction with meningeal lymphatic vessels is little by little shedding light into this conundrum. [2,6]

Until 2012, the only answer to this question lay into the classical protein degradation pathways. [7,8] The idea was that, lacking lymphatic vessels or other known exit pathways, the brain was continuously asked to recycle its own debris. The degradation of interstitial proteins can be performed by extracellular proteases, produced and expressed by astrocytes or supplied in neurons and glial cells, through the ubiquitin-proteasome pathway, the autophagy-lysosome pathway, and the endosome-lysosome pathway [9]. The discovery of the glymphatic pathway is overshadowing this assumption, opening greater perspectives in the metabolic waste clearance of the organ.

Traditionally, ISF flow was thought to be driven by diffusion. [7] However, doubts began to arise in the 1980s when Patricia Grady, injecting into the CSF of cats and dogs the specific glycoprotein horseradish peroxidase, observed that within 4-5 minutes their vascular system was extensively outlined by the tracer. Such a rapid influx of CSF into the cerebral vascular compartment could not be explained by a slow diffusion process and would have been subsequently elucidated by the convective forces that partially drive the glymphatic bulk flow [1].

The presence of a convective interstitial flow is still a matter of debate, with some researchers supporting the idea that diffusion is the dominant process. [10] These doubts are primarily based on findings from photobleaching experiments, in which fluorescence recovery shows no preferential direction, a fact that would be contrarily expected by an interstitial convective flow. While this observation suggests that diffusion could represent the main transport mechanism, it must be also considered that the process occurs at the nanoscale, where classical macroscopic physic principles describing diffusion as a passive process may not fully apply. [11]

In conclusion, it seems reasonable to assume that both convection and diffusion contribute to the fluid exchange in the brain. However, the exact contribution of each mechanism remains unclear and warrants further investigation. [12]

1.2 Cerebrospinal fluid: function, production and circulation

As previously stated, the brain is the only organ that does not have a defined lymphoid tissue. For this reason, it had to develop an equally unique mechanism to guarantee the fluid homeostasis and eliminate waste solutes. [3]

The fluid distribution in the brain follows a compartmentalization model comparable to that of the rest of the body. It consists of intracellular fluid (60-68%) and extracellular fluid (32-40%), further divided into the ISF, that envelops neurons and the CSF that surrounds the brain and the blood [2,3]. The latter is separated from the brain parenchyma by the blood - brain barrier (BBB) and from the CSF by the blood – CSF barrier (BCSFB). [2]

The BBB, as shown in *Figure 1*, is composed of endothelial cells connected by tight junctions, which prevent the paracellular movement of solutes across the vascular wall into the CNS. [3]

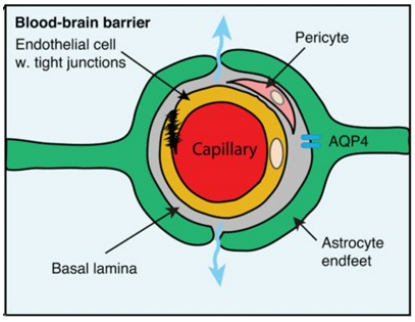

***Figure 1**: Detail from Schematic representation of the brain's fluid compartments and barriers [2]*

Conversely, the BCSFB, as depicted in *Figure 2*, is characterized by tight junctions among the epithelial cells of the choroid plexus, so that macromolecules are allowed to pass through the fenestrated endothelium but can not pass through the choroidal cells. [2]

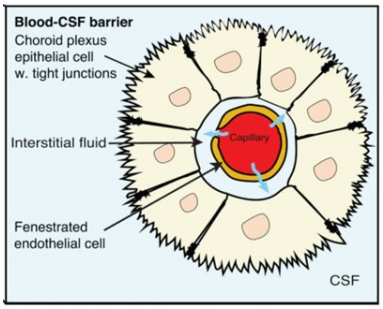

***Figure 2**: Detail from Schematic representation of the brain's fluid compartments and barriers [2]*

The choroid plexuses are highly vascularized secretory systems of ependymal epithelium that envelope the lateral, fourth and third ventricles. [2,13]. These structures form the BCSFB and play the key role in CSF production. Given the strong cohesion of choroid plexus cells through tight junctions, ions and water must travel through specific transporters located on the basolateral and apical membranes to reach the ventricular lumen. The result is a net flux of Na+, HCO3-, and Cl- across the epithelium and the simultaneous creation of an osmotic gradient, that drives water through specific conduits, the AQP4 channels. [2] Functional and morphological changes in the choroid plexuses have been observed in patients with AD, in which the accumulation of Amyloid β (Aβ) creates a proinflammatory environment populated by metalloproteinases that, weakening the tight junctions, compromise the integrity of the BCSFB. Such instances emphasizes the central role of the choroid plexuses, making them a critical area of study [14].

The CSF is a clear, colorless fluid that occupies the space between the pia mater and the arachnoid. In humans, the daily production amounts of 500 – 600 mL but its total volume is maintained at 150 mL through a consistent removal. [2,13]

It can not be considered properly an ultrafiltrate of plasma, since it is actively secreted by the choroid plexuses and presents a different electrolyte composition. [15] The CSF passes from the lateral ventricles to the third ventricles through the foramina of Monro; subsequently, it takes the cerebral aqueduct and reaches the fourth ventricle. At this point, passing through the median foramen of Magendie and the two lateral foramina of Luschka, it enters the subarachnoid space and cisterns, descending then along the spinal cord [3,13].

This fluid nourishes, protects and provides buoyancy to the CNS. [15] *Figure 3* illustrates the possible pathways it can utilize to leave the subarachnoid space: the perineural sheaths, encasing cranial and spinal nerves, the meningeal lymphatic vessels of the dura, that drive it into the cervical lymph nodes and the arachnoid granulations, which discharge into the dural sinus. [7]. The perineural CSF outflow passage through the cribriform plate has recently received particular attention: CSF drains into the nasal submucosa through the continuous lymphatic system associated with the olfactory bulbs, to be finally delivered to the cervical lymph nodes. [16] Observational studies in animal models have shown that several proteins can be identified within the nasal mucosa, including those implicated in the pathophysiology of AD. Employing brain-derived analytics in this area would open new possibilities for an earlier and non-invasive diagnosis of neurodegenerative disorders. [17]

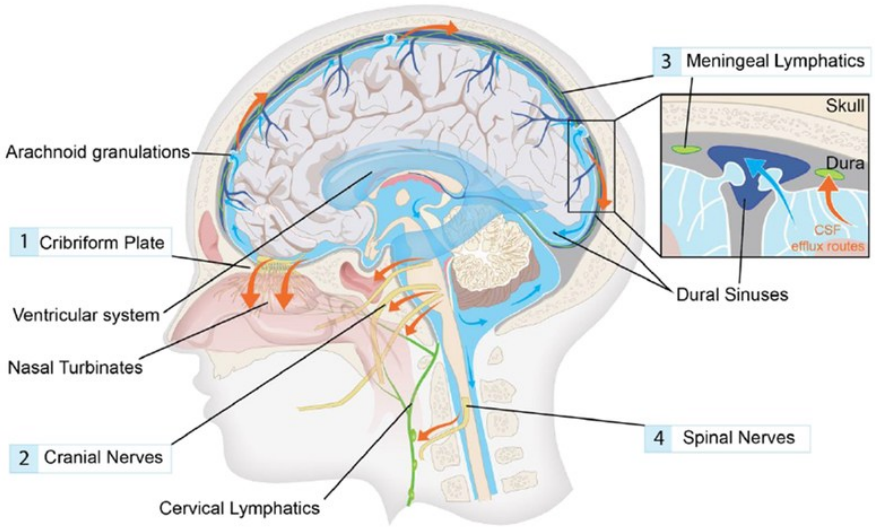

***Figure 3**: Cerebrospinal fluid efflux in humans* [7]

1.3 The architecture of the glymphatic system

The glymphatic system has been described as an intricate network of perivascular spaces, through which the CSF enters the brain parenchyma. [2,14] In the subarachnoid spaces, pial arteries bathed by the liquid turn into penetrating arteries as they dive into the brain tissue. In this section, they are enclosed within perivascular structures called Virchow-Robin spaces. These spaces are encased by a layer of leptomeningeal cells which lie adjacent to both the endothelium and the perivascular astrocyte end-feet. [2,7] As penetrating arteries further branch into

arterioles and capillaries, the PVS progressively narrow until they disappear to be replaced by the extracellular matrix (ECM), forming the basal lamina: a porous structure that provides minimal resistance to the CSF influx. [2] In addiction, perivascular and leptomeningeal macrophages, also referred to as parenchymal border macrophages (PBMs), continuously work to degrade this matrix layer and facilitate the influx of the CSF. [5]

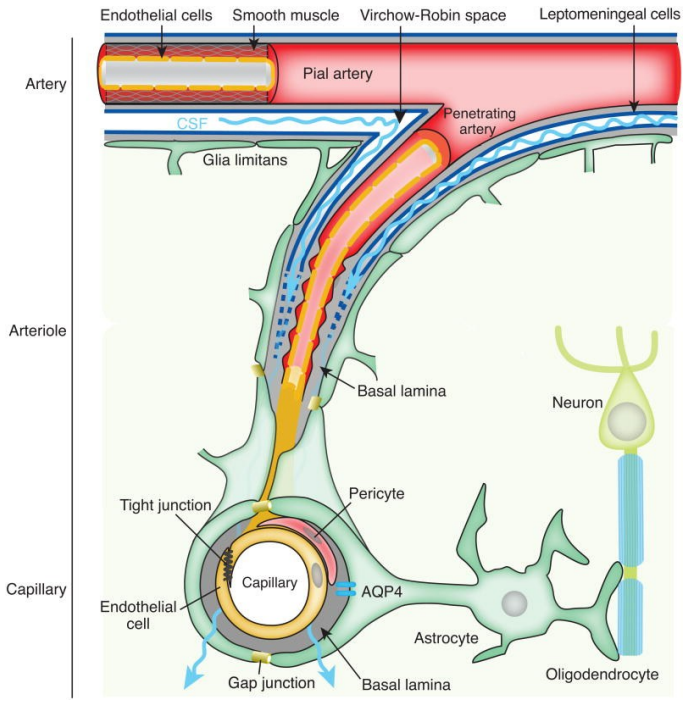

***Figure 4**: The neurovascular unit [2]*

The CSF moves deep into the parenchyma thanks to the high polarized expression of AQP4 channels along the astrocyte end-feet, that encase and define the PVS. These channels act as bridges between the perivascular and interstitial spaces of the brain, representing the essential elements of the neurovascular unit, as depicted in *Figure 4*. [3]

The pivotal role of AQP4 in the CSF-ISF exchange has been evidenced by several studies; in particular, it has been shown that AQP4 knockout mice experienced a 65% reduction in CSF fluid flux compared to wild-type animals, along with a 55% reduction in the clearance of intra-striatal injected radiolabeled amyloid. [2,3] For instance, the dysfunction of the glymphatic system observed in neurodegenerative diseases such as AD can be partially attributed to diminished polarization and altered expression of AQP4 channels. Decreased levels of AQP4

were indeed identified in the frontal lobe of deceased AD patients, whereas normal levels were detected in individuals without cognitive impairment. [18] Since the importance of AQP4 in regulating the glymphatic flow, its role and the consequences of the loss of its polarized expression will be later more thoroughly discussed.

Once inside the brain, the CSF seems to be guided by a convective flow. [5] This enables a rapid and continuous exchange between CSF and ISF, which allows the clearance of waste solutes, including Aβ and Tau, both proteins implicated in the pathogenesis of AD. [2] Moreover, the exchange between CSF and ISF has been also described as involved in the transport of other important metabolic substances, such as glucose, lipids, and the apolipoprotein E (ApoE).

The brain is well equipped to maintain homeostasis and clear excess of cholesterol, thanks to apolipoproteins secreted by the astrocytes. Part of the ApoE is additionally produced in the choroid plexuses and reaches the brain following this precise bulk flow. [14] The connection observed between a particular isoform, the ApoE4, and neurodegenerative diseases can be explained by the cooperation between this protein and the low-density lipoprotein receptor-related protein 1 (LRP1) to facilitate the clearance of Aβ. The isoform ApoE4 inhibits this interaction, unsurprisingly representing one of the genetical determined major risk factors for the development of AD. Additionally, it induces premature constriction of the meningeal lymphatic vessels, leading to reduced CSF drainage along this egress pathway. [6]

From the interstitial space the fluid flows through AQP4 channels, always located in the astrocytes end-feet, to reach this time the perivenous spaces and be transported out of the brain towards the cervical lymphatic system. [2] A schematic representation of the process is shown in *Figure 5*.

The CSF drains to the dura, thanks to the arachnoid granulations, that protrude into the venous sinus. Even though this was identified as an important egress pathway, this still left an open question: how can the direct passage of antigens and metabolites into the general circulation be related to the requirement for immune surveillance, mediated by the meningeal lymphatic stations? This dilemma is

starting to be solved thanks to the discovery of the ACE points, specialized arachnoid cuff egress sites. [5]

The bridging veins directed to the dura are enveloped by an arachnoid layer that ends in these specialized structures, populated by dural fibroblasts and different types of immune cells. These points connect the lymphatic vessels with the glymphatic flow coming from the deep brain, representing crucial points for immune regulation. Their pivotal role can be related also to the pathogenesis of waste's accumulating diseases such as AD: once they are clogged, the cleansing of toxic products from the brain can not be properly realized. [5]

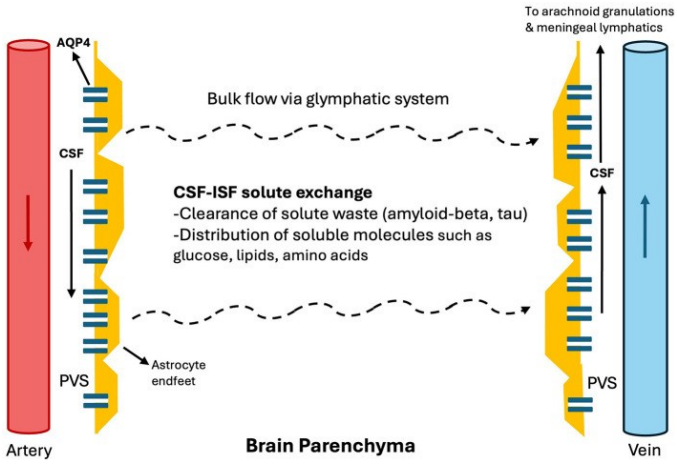

***Figure 5**: Glymphatic System [19]*

1.4 What drives the glymphatic flow?

Considering that the CSF entering the interstitial spaces infiltrates a densely populated parenchyma, to guide its flow and to allow the CSF-ISF exchange, an energy source is evidently needed. [5] This is primarily provided by the ongoing impulse in the CSF production from the choroid plexuses. [2] Yet, to clarify the mechanism that drives the glymphatic flow, it is necessary to expand the focus beyond the deep brain parenchyma and investigate its connection with the cardiovascular system.

The pulse wave generated in the ascending aorta during the systole propagates along the carotid artery and reaches the deep diving vases of the parenchyma. This pulsation enables the flow of the CSF along the PVS and its passage in the interstitial space. [8] This issue has been proved by the fact that CSF flow showed an evident increase when adrenergic agonists were administered in mice, associated

with the simultaneous growth of the pulsatility index. Contrarily, the ligation of the carotid artery induced a reduced CSF flow. [3] Not surprisingly, patients with reduced ejection fraction are at higher risk of cognitive decline. Even if this was usually thought to be caused by cerebral hypoperfusion, it seems that an impairment of the glymphatic flow could also be the culprit, or at least a contributing factor.

The relationship between blood and CSF flow can be elucidated through the Monroe-Kellie hypothesis: an increase in the volume of any of the three principal components within the CNS - blood, brain tissue or CSF - necessitates a compensatory reduction in the volume of another component; consequently, the increased vessel diameter leads to the modulation of CSF flow. [19]

On the other hand, hypertension as well play a role in the glymphatic dysfunction by stiffening the arterial walls and reducing their pulsatility. [8]

The movement of the CSF and the egress of ISF along the perivenous spaces has seen to be influenced also by the respiratory cycle. Even if these findings have not been stated in humans yet, it is reasonable to think that specific breathing patterns could lead to respiratory-related changes in the vases, influencing the flow along the PVS. [20] Additionally, animal studies demonstrated that gravity and head position play also a role in this process: mice that slept in a lateral head position had and increased flow compared to the prone-position sleeping ones. [7]

Vasomotion has also been shown to play a role in regulating the process. Parenchymal vases undergo spontaneous and continuous changes in their diameter, which are partly promoted by neural activity; animal models showed that the amplitude of these oscillations positively correlates with the increased tracer clearance. [19] This suggest that, increasing neuronal activity in specific brain regions, the fluid clearance could be there as well enhanced. [5] Notably, high-frequency gamma stimulation has been demonstrated to promote Aβ clearance in AD models, supporting the idea of potentiating the glymphatic activity as novel promising intervention target. [19]

Although neural activity partly drives the glymphatic flow, it is during sleep that this system works at its highest efficiency. This primarily happens because of the high synchronized activity that occurs in neurons during the resting state. [5]

Owing to its central role, the physiological mechanisms of sleeping and the consequences of disrupted sleep quality will be later thoroughly explored.

2. DYSFUNCTION OF THE GLYMPHATIC SYSTEM

2.1 The aging brain

Ancient philosophy and medicine embraced the idea that "Senectus ipsa est morbus" [21] – the old age itself is a disease. Aging was not simply considered a phase of life, but rather a condition of physical and mental decline that resembles a pathology. The debate on the matter is still ongoing, although modern medicine tends to consider it a natural process that leads to an increased risk of developing age-related diseases. On the other hand, those who believe that aging should be considered as a disease highlight the similarities between the changes occurring during this period and the ones observed in pathological processes.

The purpose of this paragraph is not to look deeper into this discussion, but rather to explore the influence of aging on glymphatic dysfunction; as a matter of fact, changes observed in neurodegenerative diseases are indeed similar to those observed in aging. [22]

It is widely accepted that the prevalence of cerebrovascular diseases increases with aging. Conditions such as atherosclerosis and hypertension are highly prevalent among older patients, whose vessels tend to be stiff; the result is a loss of elasticity and a decreased capacity of the brain vessels to drive the glymphatic flow. [2,23] Moreover, the signaling mediated by Notch3, which occurs in the contractility of cerebral vasculature, diminishes with age and the vascular architecture becomes more twisted, both factors contributing to reduce the glymphatic function. [24]

The aging process does not spare also the PVS, where perivascular macrophages (PVMs) become inefficient in degrading the ECM, thus increasing the resistance against CSF, as shown in *Figure 6*. [24]

The production of CSF in choroid plexuses appears also to be compromised by the aging process: choroid plexuses of elderly individuals are affected by elevated oxidative stress, present calcification and are filled by specific protein deposits referred to as Biondi ring tangles. [14]

However, the most important aspects of aging is related to the loss of AQP4 polarization. [2,20] It has been observed that old animals are affected by AQP4 depolarization, fact that contributes to accumulate Aβ and waste products. [6]

This is the direct consequence of reactive gliosis, a process that occurs during aging and leads to an increased numbers of GFAP expressing astrocytes. These glial cells tend to express AQP4 channels not only on their end feet but also along the parenchymal processes. The glial water channel disbalance play a major role in the CSF-ISF exchange impairment and is connected to an increased waste accumulation both in aging and AD. [2]

The impairment of AQP4 polarized expression is also connected to a decreased CCR7 expression, a G protein-coupled receptor that participates in the primary immune response. The consequences are immune dysregulation and an altered gene expression in blood endothelial cells, that promote an inflammatory response. [4]

The meningeal lymphatic vessels also show an impairment due to age. [6] Aged vessels have an increased diameter and branching, while the function of their valves is reduced and the permeability increased. This all leads to reduced drainage and impaired waste clearance capacity. Furthermore, along the dural lymphatic stations, age contributes to create a proinflammatory environment that further hinders the glymphatic drainage. [24]

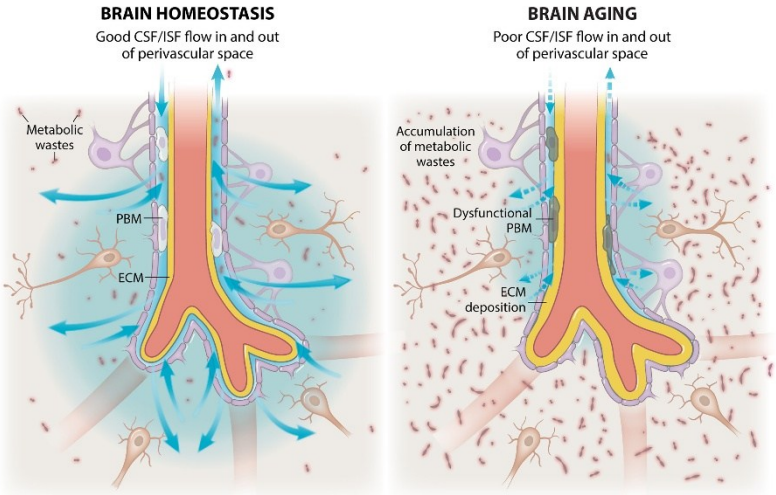

***Figure 6**: PBM dysfunction in aged brain obstructs brain fluid perfusion and impairs waste clearance [25]*

2.2 How sleep disorders impact brain health

Over an average lifetime, we spend more than 25 years sleeping. For long, it was thought that sleep was needed by the brain to rest and recover from daily activity. [25] Contrarily, EEG recordings showed that the cortical activity in REM sleep remains high and is decreased by only 15% during NREM sleep. [8] While the

reason behind spending such a significant amount of time asleep remains largely unresolved, the finding suggests that this is not merely a mechanism to conserve energy. Nevertheless, research into the glymphatic system increasingly highlights its potential role as crucial regulator of the CSF flow into the brain parenchyma.

Sleep is commonly divided into two phases and different stages. The non-Rem phase (NREM), so named because of the absence of rapid eye movements, is the first phase that occurs in sleeping and is further divided into a first stage in which there is a transition from the awake to the asleep state, a second and a third stage, characterized by the presence of sleep spindles and K complexes, as result of the increased thalamus-cortical synchronization; these synchronized oscillations are further enhanced in the fourth stage, which represents the deepest phase of sleep. This is characterized by an EEG background rhythm of slow waves, known as delta waves (0,5 -4 Hz). Subsequently, these stages are passed through in reverse, leading to the REM sleep phase, characterized by rapid eye movements and typically vivid dreaming. [25] *Figure 7* illustrates the changes in sleep architecture between young and older subjects. Elderly individuals need more time to fall asleep, sleep fewer hours and their sleep is disrupted by several arousals, all of which contribute to reduce the sleep quality. [24]

The arousal – sleep transition is related to the secretion of norepinephrine; the reduction of this neurotransmitter indicates a decreased noradrenergic activity of the locus coeruleus, that occurs when entering the sleep phase. [3] The result is an increased volume of the interstitial spaces in the brain and consequently a decreased resistance to overcome for the CSF to enter the small notches among the densely populated parenchyma. [11]

For instance, animal studies showed that natural or anesthetized induced sleeping mice had a massively increased CSF influx compared to the awake ones. [2] The anesthetic used to induce anesthesia in these subjects is dexmedetomidine, a $\alpha 2$ central receptor agonist, which inhibits the activity of the locus coeruleus, thereby diminishing norepinephrine levels and consequently the state of arousal. [2]

Since this phenomenon was observed in both natural and induced sleeping mice, it can be stated that the variation is not due to circadian rhythms but is closely associated with the state of consciousness. [3]

The essential difference between state of arousal and sleep lies in the synchronization of neural activity. Typically, during wakefulness neurons fire out of sync, creating a field potential that allows prompt responsiveness to external stimuli. During sleep, neurons progressively synchronize their firing, causing a deep wave pattern that facilitates cerebral fluid dynamic and, consequently, the removal of metabolic wastes. Additionally, during sleep vasoactive substances such as NO and several peptides are released, increasing the vascular-mediated pumping mechanism that has already been described as one of the main drivers of the glymphatic flow. [24]

Consistent with the above, it has been proved that even a night of sleep deprivation leads to Aβ accumulation, while in patients with a good sleep hygiene the clearance of Aβ is enhanced, especially during deep sleep. [4] The same results have been observed in patients affected by obstructive sleep apnea (OSA), in which fragmented sleep and reduced deep sleep, increased the intracerebral Aβ burden. [26] Exploring the connection between deep sleep and glymphatic function, which plays the key role in brain cleansing, can influence therapeutic decisions for treating sleep disorders, frequently affecting cognitively impaired patients. For example, the use of benzodiazepines has been proved to interfere with the normal sleep architecture, impairing slow wave activity, potentially resulting in this way in dysfunctions within the glymphatic system. [27]

The importance of deep sleep in widening the interstitial spaces to increase the CSF inflow and the CSF-ISF exchange has already been mentioned. However, this is not the only interaction related to the glymphatic system. Observations from animal studies found that sleep deprivation led also to AQP4 depolarization, and that reduced sleep quality decreased the expression of these channels in the mice's amygdala and dentate gyrus. [4] This connection has also been reinforced, observing that single nucleotide polymorphism of AQP4 caused a reduction of the δ waves pattern during sleep. [8]

Further studies are thus necessary to elucidate the relationship between sleep and the glymphatic system and eventually enhance the sleep-mediated clearance of waste products as new possible therapeutic target.

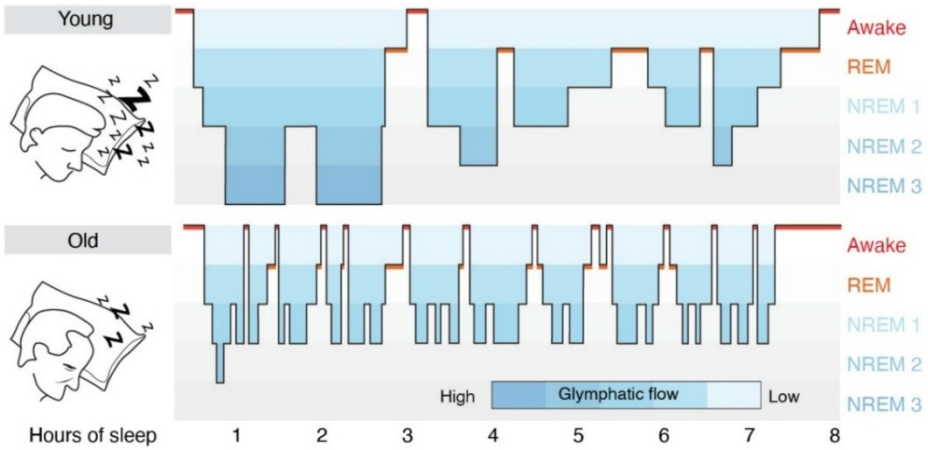

***Figure 7**: Sleep architecture in young and old subjects [8]*

2.3 Aβ accumulation: cause or consequence of glymphatic dysfunction?

Pathogenetic lesions of AD, consisting in extracellular amyloid plaques and intracellular neurofibrillary tangles, were first described in the brain of a 51-year-old patient by Alois Alzheimer in 1907. [28]

Amyloid consists in protein subunits of low molecular weight, which, misfolded and consequently insoluble, tend towards extracellular accumulation. [29] These deposits are derived from the Aβ protein, a peptide of 39 - 43 aminoacidic residues generated through the cleavage of a precursor protein. (For a deeper explanation of this process see paragraph 1.4 A glimpse into AD: from pathogenesis to clinical features). [30] When Amyloid-β deposits and aggregates in plaques in the parenchyma, the result is the development of AD, while if it accumulates at the cerebrovascular levels, this results in a cerebral amyloid angiopathy (CAA). [31]

The deposit of these proteins causing the two mentioned conditions has been related to a reduced clearance rather than an increased production of the peptide. Purification from Aβ is ensured by several processes, among which the recently described glymphatic flow seems to play the key role. [31]

However, the intramural periarterial drainage, another important pathway of Aβ clearance, needs as well to be mentioned in this paragraph. This encompasses a process through which the Aβ produced in the parenchyma exits through the walls of the arterioles, travelling in the opposite direction of blood flow. As depicted in *Figure 8* the accumulation of Aβ between smooth muscle cells and the basal

membrane results in impaired vasoactive activity and a self-reinforced reduced Aβ clearance, both in AD and CAA. [19,31]

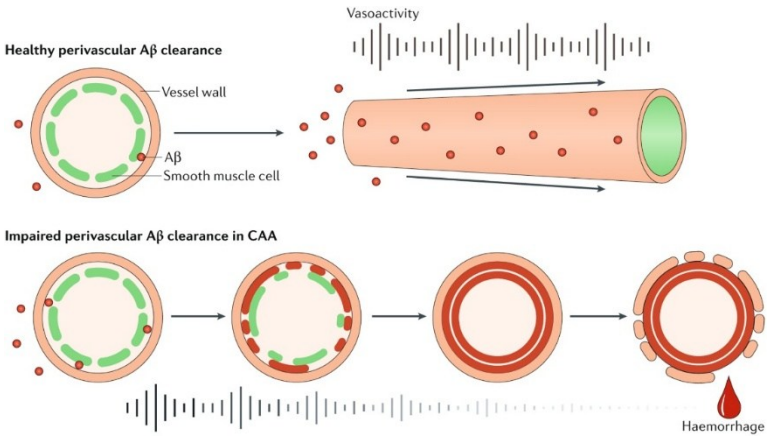

***Figure 8**: Impairment of perivascular drainage in cerebral amyloid angiopathy and Alzheimer disease [31]*

This peptide is produced by neurons and oligodendrocytes particularly during wakefulness and its release decreases during sleep, along with the reduction of neural activities. [2] Physiologically, this peptide is involved in synaptic regulation and in the survival of neural cells. [7]

As already seen, several animal studies proved the strong relationship between Aβ deposition and glymphatic impairment. Additionally, it was also stated that wildtype mice, pretreated with Aβ, showed a reduced CSF influx as in double transgenic APP/PS1 mice. This finding implies that amyloid deposition and glymphatic dysfunction should be interpreted within a bidirectional framework, instead of considered as a simply cause-and-effect chain. [3] As a matter of fact, glymphatic disfunction leads to stagnation and this promotes proteins aggregation. [8] A vitious cycle is thus triggered: Aβ accumulation in the perivascular district disrupts the drainage pathway, which in turn exacerbates its burden in PVS and interstitial spaces, ultimately facilitating the parenchymal aggregation into plaques. [26]

It has been proposed that aggregated proteins may spread via a prion - like mechanism through synaptic connections. However, the observation that their diffusion occurs both in anterograde and retrograde directions casts doubt on this hypothesis. It is more likely that this prion-like mechanism occurs through the extracellular space, driven by the glymphatic flow, rather than along synaptic

connections. In this way, when the flow stagnates in a specific region, the local burden of Aβ increases as well, further promoting its deposition. [8]

Although Aβ plaques represent an early hallmark of AD, neurodegeneration and cognitive decline in AD patients seem to be more associated with the subsequent tau pathology and glial activation. [8,32]

As previously mentioned, the pathogenesis of AD also includes the intracellular accumulation of hyperphosphorylated tau, which forms neurofibrillary tangles. [32] Glymphatic drainage dysfunction has been as well linked to both the onset and progression of tau pathology. [6] It has been in fact demonstrated that CSF-ISF exchange plays an essential role in tau clearance: areas with reduced CSF flow had increased tau accumulation. Furthermore, the relationship between the absence of AQP4 and increased Aβ burden - strongly demonstrated by Iliff *et al.*-, can also be extended to tau pathology. Specifically, reduced tau clearance was observed both when AQP4 was pharmacologically inhibited and expressed with a depolarized pattern, as seen in age-related and AD-related reactive gliosis. [32]

Returning to the question whether glymphatic dysfunction precedes or follows amyloid accumulation, it is now evident that the answer cannot be reduced to a simple causal relationship. Rather, the two processes appear to interact in a bidirectional manner reinforcing each other. Specifically, the presence of Aβ in the PVS, consequence of glymphatic impairment, further causes dysfunctional drainage and enlarges the downstream PVS, as illustrated in *Figure 9*.

Notably, the enlargement of the Virchow-Robin spaces, a hallmark of glymphatic dysfunction, may represent an early diagnostic marker of AD. [31,33]

The connection between compromised glymphatic clearance and waste accumulation could in this way predict the onset of neurodegenerative diseases. In this context, the advancements in MRI techniques, based on diffusion tensor image (DTI) to calculate a specific index, which studies the diffusion along the perivascular spaces (ALPS-index), are emerging as promising techniques for investigating glymphatic dysfunction during the prodromal phase of AD. [34]

Given the relevance of this topic, a dedicated chapter will focus on its detailed discussion.

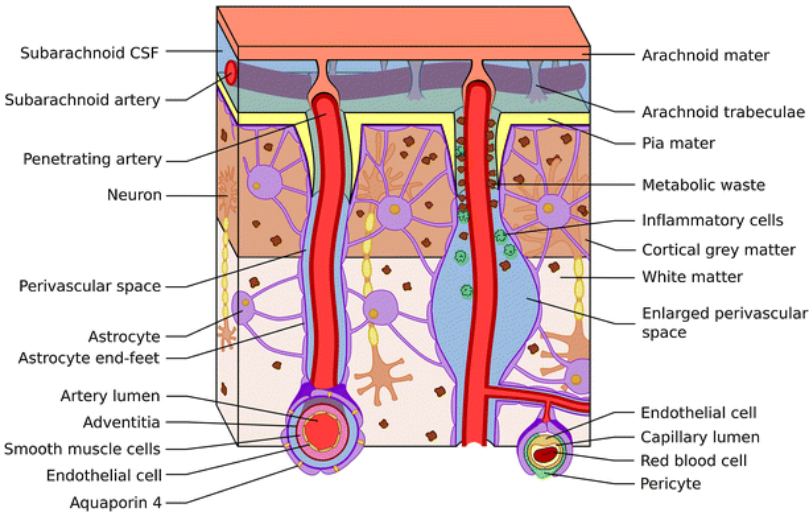

***Figure 9**: Schematic representation of the brain's perivascular Virchow–Robin space and surrounding tissues [35]*

2.4 Glymphatic impairment as a common pathway to dementia

Neurodegenerative diseases are usually associated with the accumulation of waste products in the brain. Given that the glymphatic system represents an efficient clearance pathway, it appears evident that its dysfunction contributes to the onset of these conditions and the exacerbation of their clinical manifestations. [2]

Recent studies have indeed suggested that the glymphatic system impairment may play a major role in several neurodegenerative disorders.

Although these diseases differ in the specific substances that accumulate, the aggregation mechanisms of the debris often share a common feature, linked to the interaction between regions rich in β-sheets structures. This results in the facilitated aggregation of monomers, which then tend to recruit other proteins. [8] For example, plaques identifiable in AD in addition to Aβ proteins contain also complement proteins, metalloproteinase 9, glycosaminoglycans, and apolipoproteins. [31]

For instance, Aβ accumulation is not limited to AD; it can also be found in idiopathic normal pressure hydrocephalus (iNPH), a condition characterized by the triad of gait disturbance, urinary incontinence and cognitive decline, that enters in the differential diagnosis of AD. Thanks to imaging analysis along the perivascular

spaces and the derived ALPS-index, recent studies demonstrated that this ratio was reduced in iNPH patients compared to the healthy ones, highlighting that a glymphatic impairment occurs also in this condition. [26]

The disfunction of CSF-ISF exchange has also been proposed as a potential contributing factor to the α-synuclein accumulation in Parkinson disease (PD). The real role of glymphatic dysfunction in pathologies characterized by decreased dopaminergic levels as PD is not fully understood; however, it has been observed that in mice affected by an overexpression of α-synuclein, a decreased AQP4 expression further enhanced its deposition, leading to the subsequent dopaminergic neuronal loss. Moreover, sleep disorders - frequently observed in PD - may potentially exacerbate the glymphatic dysfunction, providing another connection between α-synuclein clearance and glymphatic function. [4,24,27]

An impairment of the diffusion along PVS has been as well observed in patients affected by vascular dementia. Studies conducted in animal models showed indeed that, by injecting cholesterol crystals into the carotid artery, the resultant cognitive impairment was associated with PVS enlargement and AQP4 unpolarized expression, mirroring the pathological changes that occur in AD. [7]

Similar findings were reported in patients that experienced traumatic brain injury and then developed a cognitive impairment similar to those affected by AD or PD. [4] This phenomenon has been attributed to the massive release of Aβ and cleaved tau (C-tau) proteins that followed the injury. Tau proteins accumulate in the interstitial space and are then internalized by neurons, where they contribute to the formation of neurofibrillary tangles. This process is exacerbated by the contextual dysfunction of the glymphatic system. Specifically, the formation of glial scars initiates a reactive gliosis process, resulting in the dislocation of AQP4 channels from the astrocytes end feet to their parenchymal processes. These changes resemble those observed both in aging and AD, reinforcing in this way the idea that glymphatic impairment contributes to neurodegeneration and cognitive decline across different etiologies. [2]

3. IMAGING OF THE GLYMPHATIC SYSTEM

Inscribed on the temple of Apollo at Delphi, the aphorism "Γνῶθι σεαυτόν" (*gnōthi seautón* – know yourself) prompts reflection on the human attempt to explore the nature of self as early as the dawn of Western medicine. The philosophical question that still engages modern thinkers such as Arnaldo Benini is whether the human mind is capable of studying the brain structures from which itself arises. [35] Though the paradox remains unresolved, recent developments in neuroscience have offered new insights into the physical substrate of the mind; modern imaging techniques provide a direct window into brain activity, helping to elucidate even deep processes, such as the mechanisms driving glymphatic clearance.

Glymphatic imaging employs *ex vivo* and *in vivo* modalities, which are summarized in *Figure 10*; *ex vivo* techniques utilize brain sections derived from animals, which are pretreated with a CSF tracer. By using simultaneously immunohistochemistry techniques, it is also possible to map the AQP4 expression and relate it to the CSF tracer deposition. [7]

The glymphatic system was initially described using the two-photon microscopy. This *in vivo* imaging technique is based on the concurrent absorption of two photons by a fluorescent molecule, when and where hit by the pulsed infrared laser. By injecting a fluorescent tracer into the CSF, it is thus possible to visualize and track in real time fluid dynamics within the CNS. [2,36] To examine the glymphatic flow in both the cortex and the entire brain, alternative *in vivo* methods include transcranial macroscopic imaging, as well as MRI and PET/CT scans. [7]

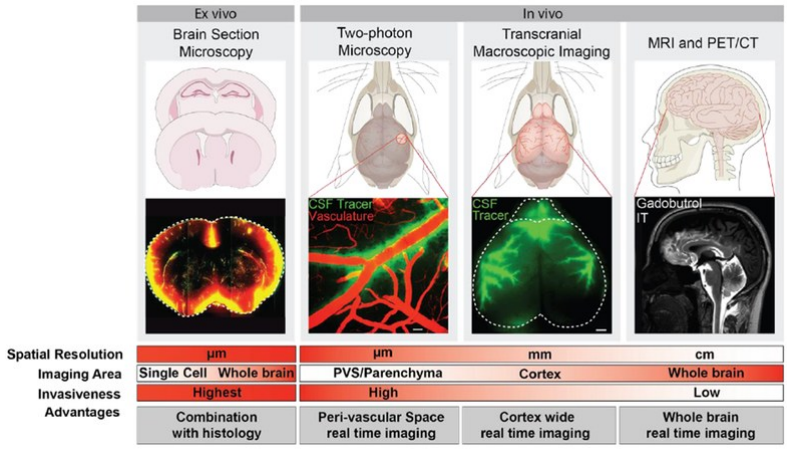

***Figure 10**: Glymphatic imaging modalities [7]*

In 2017 Taoka *et al*. proposed to quantify the activity of the glymphatic system through the diffusion tensor imaging analysis along perivascular spaces (ALPS). The resulting index, known as ALPS-index, represents a non-invasive way to describe perivascular diffusion, by assuming that at a minimum diffusion level, the index is close to 1, while when perivascular diffusion grows up, the index rises as well. [34,37]

Diffusion-tensor imaging (DTI) consists of magnetic resonance imaging (MRI) weighted sequences (DWI), that allow to quantify the rate and the direction of water diffusion. The influence of brain architecture towards water diffusion is described by fractional anisotropy, a number from 0 to 1, where 0 represents a completely unrestricted diffusion process and 1 stands for a unidirectional forced movement. [38]

This process can be represented using a tensor: a mathematical construct that allows the three-dimensional evaluation of diffusion, according to changes in fractional anisotropy. Within a voxel of an axon, which is the smallest three-dimensional unit of the tissue, this model allows the determination of the three eigenvalues λ1, λ2 and λ3, from which the three main diffusion measures can be obtained: mean diffusivity, axial diffusivity and radial diffusivity. The Aβ accumulation along axons observed in AD leads to restricted diffusion parallel to myelinated fibers, which is primarily reflected by a decreased axial diffusivity. [38]

The ALPS-index is calculated within specific regions of interest (ROIs), considering the presence of projection, association and subcortical fibers and three possible axes of diffusion: x axis (right - left), y axis (anterior - posterior) and z axis (inferior - superior).

To describe the diffusion along the PVS, which reflects the glymphatic flow, diffusivity along x-axis in projection and association fibers is the most indicated measure, thus entering as numerator of this index. Subcortical fibers are not considered since they are aligned parallel to the PVS and this fact would challenge the assumption whether the diffusion rate refers to those or to the PVS. As denominator, the ALPS ratio includes orthogonal diffusivity, measured along the y-axis for projection fibers and the z-axis for association fibers, as shown in the formula below. [34]

$$ALPS = \frac{\text{Dxx,proj+ Dxx,assoc}}{\text{Dyy,proj+ Dzz,assoc}}$$

This equation considers hypothetically that there are no crossing fibers, while as depicted in *Figure 11 D* in some ROIs the association fibers are crossed by other fibers, representing potentially a confounding factor that must be considered in order not to overestimate the ALPS index in these areas. [37]

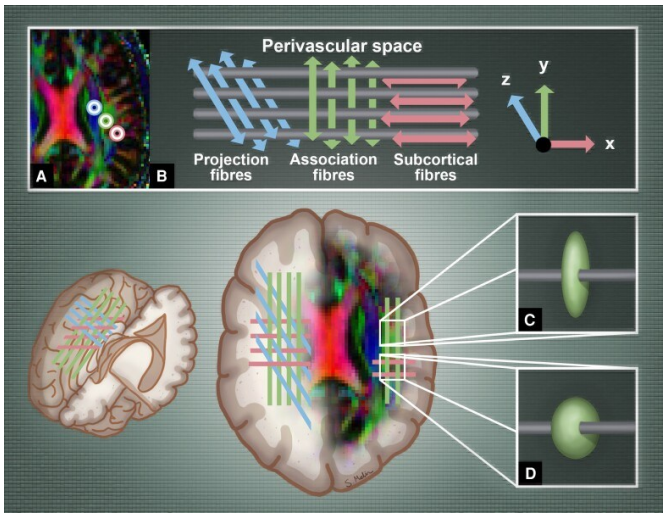

***Figure 11**: Schematic representation of the main fibers at the lateral ventricular body, illustrating how crossing fibers affect the diffusion tensor [38]*

Even if this ratio can be influenced by confounding factors such as the presence of the above mentioned crossing fibers, a recent meta-analysis, aimed to evaluate its robustness in describing the glymphatic impairment in AD and PD patients, concluded that this is a reliable index and that it might be included in the diagnostic process of neurodegenerative diseases, also considering its non invasiveness. [39]

As subsequently illustrated in *Figure 15*, this index may indeed emerge as a promising biomarker, offering a new interpretative framework of the classical amyloidogenic theory, considering the impaired glymphatic clearance an upstream event in the neurodegenerative cascade.

4. ALZHEIMER DISEASE

"It may have been a time of listening to music, a time of looking at the door of a dance hall [...] a time of hearing your father and mother singing Christmas carols." [40] With these words Penfield described the incredible capacity of the brain to keep track of past experiences, that he managed to reawake as vivid memories through electrical stimulation. The impressive recording ability of our brain shapes our identity, encompasses our past and present, representing one of the most fascinating aspect of the physiology of the self. [41]

This remarkable ability gradually disappears in neurodegenerative diseases such as AD, where the progressive loss of episodic and autobiographic memory is one of the most known and impairing aspects; even more so, it cannot be considered just a clinical symptom but should be more properly interpreted as the evident sign of a fading identity.

One of the most famous examples of this process is the series of self-portraits painted by William Utermohlen, an American artist who was diagnosed with AD. The progressive simplification of his image reflects the cognitive decline caused by the disease, which ultimately leads to the complete loss of self perception as shown in *Figure 12*. [42]

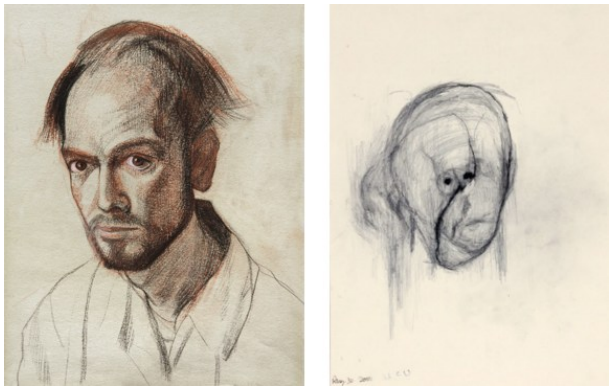

***Figure 12**: Self portrait 1967 (left) ; Head I 2000 (right) [43]*

4.1 A glimpse into AD: from pathogenesis to clinical features

Among all several cases of cognitively impaired patients, 50-60% are attributed to AD, which indeed represents the primary cause of dementia. As the population continues to age and advanced age is one of the main risk factors for its development, it is evident that the number of people with a cognitive decline,

including those affected by AD, will also increase, with an overall prevalence estimated to exceed 130 million cases by 2050. [28,43] The incidence is higher in women than in men, fact traditionally attributed to their longer average lifespan and the decrease of estrogen levels after menopause. [28]

The probability of developing the disease during life is influenced by multiple aspects that include environmental and lifestyle factors, such as smoking, poor sleep quality, obesity, physical inactivity, social isolation but also genetic mutations. The isoform ε4 of APOE has already been described as a possible predisposing condition (see paragraph 1.3 The architecture of the glymphatic system); however, the most important genetic causes of AD are associated with mutations in APP, PS1 and PS2 genes, which are responsible for the majority of early-onset AD cases, affecting individuals under the age of 65 years. [44]

This genetical predisposition can be explained according to the "amyloid hypothesis" illustrated in *Figure 13*: β-Amyloid Precursor Protein (βAPP) can take a non-amyloidogenic or an amyloidogenic pathway according to the difference cleavage performed by the α-secretase, β-secretase or γ-secretase proteins. Mutation of the above mentioned genes, that respectively encode for APP and presenilin 1 or presenilin 2 - fundamental element of the γ-secretase complex - promote the final release of Aβ, which, especially if formed by 42 amino acids, is the highest amyloidogenic form of the peptide. [44,45]

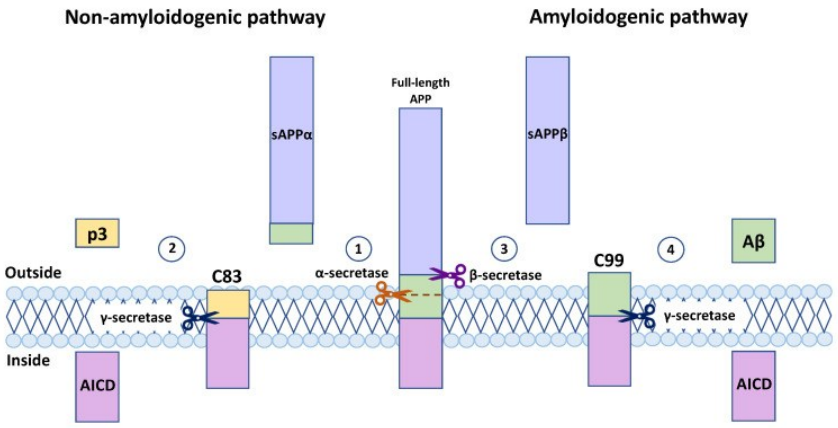

***Figure 13**: Amyloid precursor proteins processing pathways [46]*

This amyloidogenic process leads to the formation of extracellular soluble oligomers that tend to aggregate into plaques, thus representing one of the typical hallmarks of AD. Notably, Aβ42 shows a greater tendency to build up parenchymal insoluble fibrils compared to Aβ40, that tends to accumulate in vases, developing CAA. [31,45]

Another hallmark of the disease is the presence of intracellular neurofibrillary tangles of hyperphosphorylated tau. This protein usually stabilizes axonal microtubules, but upon hyperphosphorylation, it loses its function, compromising consequently the axonal transport. At a macroscopic level, the neurodegenerative process is characterized by cortical atrophy, primarily involving the mesial temporal regions and the hippocampus. [28]

In the last revision of the AD diagnostic criteria it is stated that the existence of the disease is closely linked to the underlying neuropathological process, rather then to clinical manifestations. [46] However, clinical evaluation still plays the key role in distinguishing between overt dementia and mild cognitive impairment (MCI). The latter is described as a deficit in at least one cognitive domain, which does not hinder the routine daily activities. [44]

In the early stage of the disease the patient is affected by a short term and episodic memory impairment, which may subsequently cause a reactive form of depression. Additionally, language production can be impaired by delayed lexical access and disrupted speeches may obstacle the communication ability. With the progression of the disease other cognitive functions get lost and visuo-spatial deficits, apraxia or behavioral changes can emerge; in late-stage AD patients the long-term memory is also affected and the cognitive deterioration is accompanied by a physical decline, that causes the death of the patient within 8-10 years. [28,44]

4.2 Beyond AT(N): reconsidering the role of glymphatic dysfunction

The current AD diagnostic criteria are based on the AT(N) classification scheme. “A” stands for Aβ pathology and is classified as present/abnormal (+) if Aβ42 in the CSF is decreased, the CSF Aβ42/40 ratio is decreased or by amyloid PET positivity; “T” refers to phosphorylated tau pathology (ptau), that can be detected through elevated CSF ptau levels or positive tau PET imaging; “N” stands for neurodegeneration or neuronal injury, which, considered a non-specific AD

biomarker, can be determined by increased levels of neurofilament light chain (NfL) in the CSF, MRI images showing brain atrophy or hypometabolism detected using a FDG-PET. As depicted in *Figure 14* these biomarkers can be utilized for an early diagnostic of AD, before the onset of clinical symptoms, indicated as "C" in the figure. [46]

Recently, studies on the glymphatic system have suggested that its dysfunction may serve as a promising new biomarker in the diagnostic process of the disease.

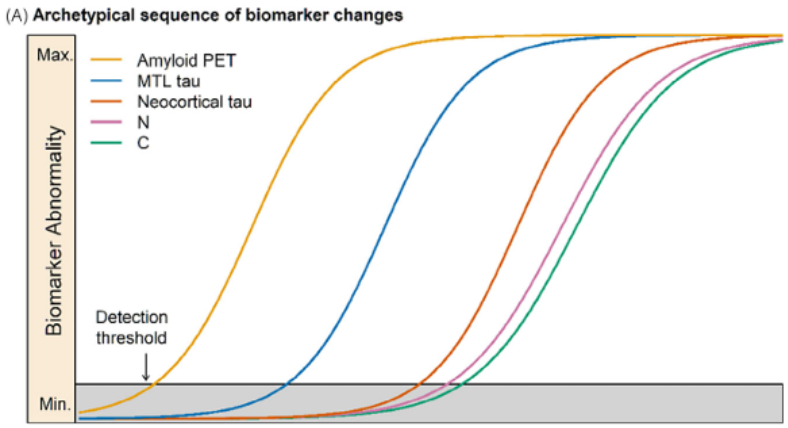

***Figure 14**: Staging illustrated with imaging biomarkers [47]*

An indirect way to evaluate the glymphatic system is to quantify the PVS enlargement. [47] Their widening could in fact mirror a structural change due to the accumulation of waste substances, caused by the dysfunctional drainage.

This issue is supported by a three-year longitudinal multicenter study that examined the PVS dynamics in healthy aging patients and AD individuals. As previous studies demonstrated, aging itself leads to the enlargement of these spaces, although the presence of Aβ and tau in AD cases was associated with an acceleration of this process. [33]

Similarly, the ALPS index, already mentioned as a reliable indicator of reduced glymphatic function, could also represent a promising early biomarker. Consistent with this, a pioneering study demonstrated that the reduction of the ALPS index precedes Aβ deposition, as quantified through amyloid PET imaging and CSF analysis, potentially representing the first detectable alteration in the AD pathological cascade. Moreover, a decrease in the ALPS index seems also suitable to predict the risk of progression from asymptomatic stage to MCI and AD

dementia. *Figure 15* sums up the new possible sequence of biomarker changes, wherein ALPS reduction becomes the earliest detectable predictor. As shown in the figure, after the initial decline, the DTI-ALPS index reaches a plateau phase; afterward, as the disease progresses to severe stages of dementia, the index worsens again, mirroring the further glymphatic dysfunction associated with the increased amyloid burden.

Although this study supports the predictive role of the ALPS index on amyloid accumulation and brain atrophy, further researches are necessary to elucidate this association and establish a possible causal relationship. [48]

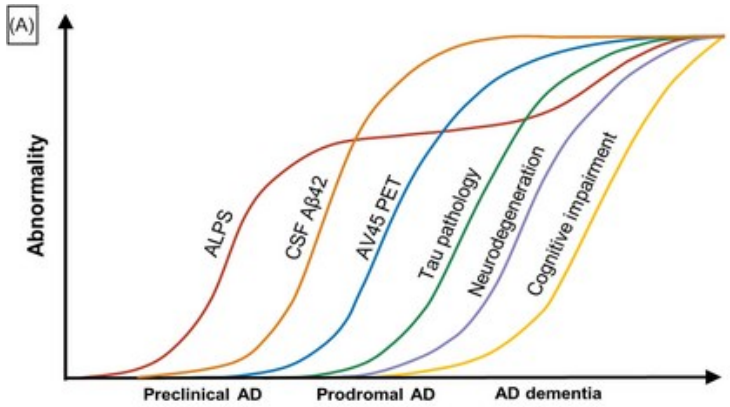

***Figure 15**: ALPS index predicts cognition change through amyloid and neurodegeneration [49]*

4.3 Current developments and future challenges in AD therapy

Current pharmacological therapies for AD consist in cholinesterase inhibitors such as donepezil, rivastigmine and galantamine, which aim to restore the reduced cholinergic transmission observed in AD, and on memantine, a N-methyl-D-aspartate (NMDA) receptor antagonist, which protects the brain from glutamate-induced excitotoxicity. Although these drugs provide some symptomatic relief and modest cognitive benefits, they do not modify the progression of the disease. [49]

In contrast, anti-amyloid drugs are emerging as promising disease-modifying therapies. Just as Rituximab revolutionized the treatment of chronic lymphocytic leukemia (CLL), there is hope that Lecanemab, targeting the protofibril forms of Aβ, or Donanemab, a plaque specific antibody, will be as well remembered as the first effective therapies able to change the natural history of AD. [50]

These second-generation anti-amyloid antibodies have been recognized as capable of reducing the parenchymal accumulation of Aβ. Historically, Aducanumab was the first drug of this class approved by the FDA, though it has been withdrawn from clinical use due to limited evidence of clinical efficacy and significant safety concerns, related to amyloid-related imaging abnormalities (ARIA). Gantenerumab, another second-generation monoclonal antibody, on the other hand, did not reach the primary efficacy endpoints in clinical trials. [17,50]

Recent studies demonstrated that both Lecanemab and Donanemab were able to slow down cognitive decline in MCI or early AD patients. Nevertheless, their efficacy is also accompanied by the already mentioned ARIA, side effects detectable with MRI techniques which include vasogenic edema (ARIA-E) and microhemorrhages (ARIA-H). [17,50]

Future challenges in AD therapies are connected to the so-called ARIA – paradox: the more effective a treatment is at clearing amyloid, the higher is the risk of developing ARIA. Although the exact underlying mechanism remains not fully understood, it is reasonable to think that the antibody–Aβ interactions trigger an excessive immune response via the classical complement pathway. This result in the formation of immune complexes and their accumulation along PVS, which impair the IPAD and damage the vascular integrity. Furthermore, abnormalities in the CSF – ISF exchange may also contribute to ARIA pathogenesis. [17,50]

These findings highlight the need to develop drugs with a high efficacy in achieving the desired clinical endpoints, but at the same time associated with a lower risk of ARIA adverse events. In this context, a deeper understanding of the glymphatic system and its role in Aβ clearance could offer important insights: investing how glymphatic dysfunction contributes to impaired Aβ elimination and potentially to the development of ARIA may indeed open new possibilities for both diagnostic and therapeutic perspectives of AD.

OBJECTIVES

Glymphatic dysfunction in AD cases is thought to play a key role in initiating the pathological cascade that leads to the accumulation of waste products and consequently to neurodegeneration and clinical manifestations of dementia. [51]

This concept may be extended to other neurodegenerative diseases such as PD. In this regard, McKnight *et. al* proposed that alterations in perivascular fluid dynamics underlie α-synuclein accumulation in PD , analogous to amyloid-β accumulation in AD. [52]

The impairment of the glymphatic clearance seems to clog up the system upstream, creating a vitious cycle that further reduces the perivascular drainage, increasing the accumulation of waste products and causing the enlargement of the perivascular spaces.

These alterations occurring in the perivascular pathways enable us to study the glymphatic system in vivo. The enlarged PVS are detectable using MRI techniques and changes in water diffusivity can be assessed using DWI-MRI sequences and further quantified by the DTI-ALPS index as proposed by Taoka et al. [47,53]

Although the DTI-ALPS index appears to be a promising proxy for assessing glymphatic dysfunction, studying the glymphatic system remains challenging and the index itself has certain limitations. Therefore, it is important to explore additional accessible methods to quantify the activity of this system. For example, Sacchi *et al.* proposed to measure the AQP4 levels in the CSF of cognitively impaired individuals and found elevated levels particularly in patients diagnosed with neurodegenerative diseases, especially AD. Such approaches could complement imaging-based methods and contribute to a more comprehensive evaluation of glymphatic impairment. [47]

Moreover, the relationship between the degree of glymphatic dysfunction and clinical severity remains unclear. Evidences suggest that this drainage system may be impaired in the earliest stages of the disease, even before overt clinical symptoms emerge, when cognitive difficulties are only detectable using neuropsychological assessments.

We investigated differences in the glymphatic system according to presence/absence of brain parenchymal amyloid deposition and the association between DTI-ALPS index values and structural network alterations in people with different types of dementia testing positive or negative to Amyloid PET.

The aims were as follows:

a) To assess whether DTI-ALPS values were associated with amyloid positivity in different subgroups of patients with neurocognitive decline due to different neurodegenerative diseases (Alzheimer disease spectrum, synucleinopathies, frontotemporal dementia);
b) To study a correlation of DTI-ALPS values with demographic and cognitive data;
c) To investigate the implication of altered glymphatic system on disruption of network-based structural connectivity.

MATERIALS AND METHODS

1. Study population

This monocentric longitudinal study enrolled 151 subjects who underwent DWI and amyloid PET scans in a PET/MR integrated system between 2013 and 2024. For this study, neuroimaging analyses have been performed on 104 subjects.

1.1 Inclusion and exclusion criteria

Patients were eligible for the inclusion in the study if they met one of the following conditions:

- cognitive impairment with a diagnosis of AD (MCI or dementia);
- cognitive impairment with a diagnosis of non-Alzheimer dementia of neurodegenerative type (i.e. FTD, PD/PDD/DLB);
- neuroimaging evidence of cerebral amyloid angiopathy;
- diagnosis of mild cognitive impairment with negative amyloid PET (exclusion of AD, not established a definite alternative diagnosis).

Exclusion criteria included:

- the presence of intracranial space-occupying lesion (e.g. brain tumors);
- alcohol and drugs abuse;
- contraindications to PET/MRI imaging, including MRI-incompatible pacemakers, pregnancy or other conditions incompatible with tracer injections;
- inability to complete the study protocol;
- unavailable minimum dataset of clinical information.

Subjects were also excluded from the analysis in the case of:

- Poor imaging quality;
- Undetermined diagnosis.

In addition to the assessments needed to establish or exclude these diagnoses (i.e. CSF analysis, PET-FDG, DAT-SCAN, genetic panels, neuropsychological evaluations) all included patients had available a brain 3T scan and a co-acquired amyloid PET imaging with either flutemetamol or florbetaben tracer.

All the subjects provided informed consent for the use of their clinical data in anonymous form to research purposes.

The diagnostic work-up for each patient was based on a comprehensive medical history, general and neurological examinations, neuropsychological assessments and neuroimaging. Most of the patients were clinically followed at the Neurological Clinic and CRIC (University Hospital of Padova, Italy) from 2013 to date. A few patients were recruited from other institutions. Clinical data of these patients were obtained with access to available medical records. For the patients recruited from other institutions, access to detailed medical records was limited and the only available data were the diagnosis and the clinical severity at the time of PET imaging.

Out of 104 who met inclusion criteria, 86 survived exclusion criteria and had images of sufficient quality for further analysis. Among these, one patient was excluded due to a diagnosis susceptible to interpretative bias (co-pathology with overlap AD/FTD).

The final study cohort included 85 patients (31 females and 54 males) aged between 43 and 87 years, with a mean age of 67,39±8,07 years.

1.2 Diagnostic classification

The diagnostic classification of the analyzed cohort is summarized in *Figure 16*.

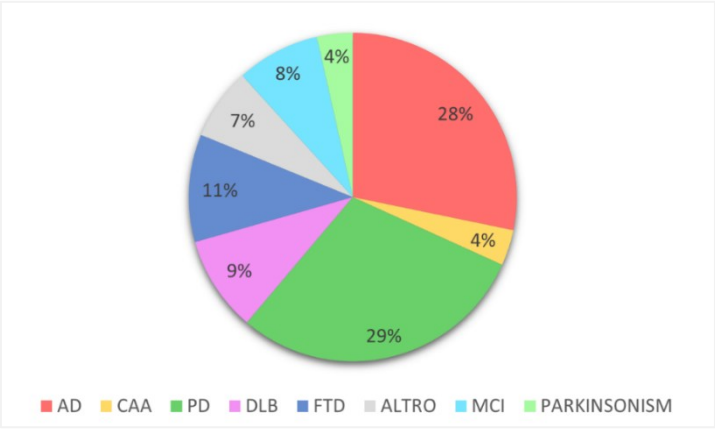

***Figure 16**: Diagnostic classification of the patients considering the whole cohort*

Twenty-four patients received a biological diagnosis of AD, in accordance with the most recent diagnostic criteria. [46] The non-AD patients were clinically classified as follows:

- Parkinson disease (PD), including PD-MCI and Parkinson's disease dementia (PDD), n=25;
- Atypical parkinsonism, including progressive supranuclear palsy (PSP), multiple system atrophy parkinsonian type (MSA-P) and corticobasal degeneration (CBD), n=3;
- Frontotemporal dementia (FTD), n=9;
- Cerebral amyloid angiopathy (CAA), n=3;
- Dementia with Lewy bodies (DLB), n=8;
- Mild cognitive impairment (MCI), including non-amnestic (naMCI) and subjective subtypes (sMCI), n=7;
- Other conditions, including cerebrovascular disease, primary psychiatric disorders (PPD), limbic-predominant age-related TDP-43 encephalopathy (LATE) and chronic traumatic encephalopathy (CTE), n=6.

Patients were further stratified according to the clinical variants of the disease. The distribution by clinical variant is illustrated in *Figure 17*.

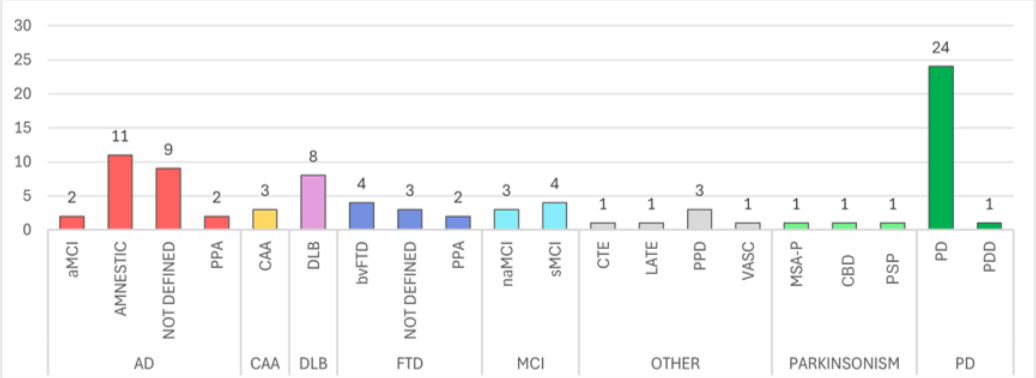

***Figure 17**: Diagnoses stratified by clinical variants*

1.3 Complementary diagnostics

Within the cohort, 41 patients additionally underwent FDG-PET imaging, 22 patients had CSF biomarkers, and 11 patients underwent DAT-SPECT imaging. Moreover, 37 patients completed a second-level neuropsychological assessment.

1.4 Pharmacological treatment

Pharmacological treatment data at the time of PET imaging were available for 51 of the 85 patients. Treatments included symptomatic dementia therapies,

antidepressants, anxiolytics, antipsychotics and medications for PD. The distribution of treatments is shown in *Figure 18*.

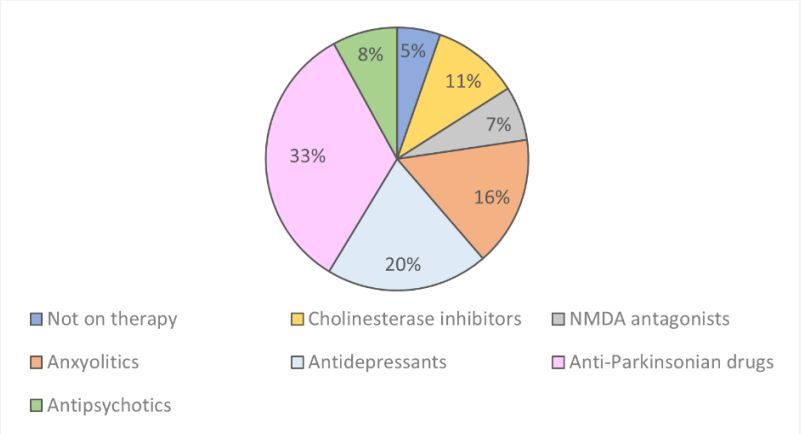

***Figure 18**: Pharmacological treatment at the time of PET acquisition*

1.5 Recruitment

Patients were recruited from different institutions: the Neurology department of Padova, the Regional Center for Brain Aging (CRIC) and other clinical services. The number of patients sent by each institution is summarized in *Figure 19*.

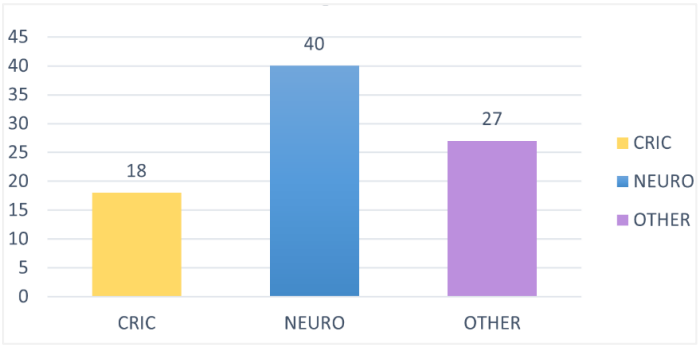

***Figure 19**: Sending institutions*

1.6 Amyloid status and cognitive profiles

Based on amyloid PET results, patients were classified in amyloid-positive (AMY+, n=42; 16 females; mean age 68.16 ± 7.07 years) or amyloid-negative (AMY−, n=43; 15 females; mean age 67 ± 9 years).

Cognitive severity was assessed using the Mini-Mental State Examination (MMSE) and showed comparable results between the two groups: the AMY+ group presented a mean MMSE score of 21.86 ± 4.29, while the AMY− group had a mean score of 23.76 ± 6.06 (t= 1,43; p=0,16).

In some cases, MMSE scores were estimated using the Montreal Cognitive Assessment (MoCA) equivalents, according to validated conversion tables. [54]

For 23 patients, no MMSE evaluation was available near the time of PET imaging.

2. Imaging techniques: a comprehensive overview

All patients underwent brain MRI and amyloid PET scans using a 3T PET/MR integrated system (Siemens Biograph mMR).

2.1 Amyloid PET and radiotracers

Amyloid PET imaging was performed using two different radiotracers: [^{18}F] florbetaben (FBB) was administered to 39 patients and [^{18}F] flutemetamol (FMM) was used in 46 cases.

Indications for amyloid PET imaging include persistent or progressive unexplained MCI, the presence of core AD biomarkers in atypical or mixed clinical presentations and early-onset dementia. [55] In cases where CSF biomarker results are borderline and the clinical suspicion of AD persists, a positive amyloid PET scan enables confirmation of an etiological diagnosis. [56]

Images interpretation requires trained readers who visually assess tracer uptake. A scan is considered positive, if the binding occurs in one or more cortical brain regions and negative, if the uptake is manly confined to the white matter. When visual interpretation is ambiguous, amyloid quantification techniques can improve the diagnostic accuracy and categorize the amyloid burden in low, intermediate or high, thus overcoming the limitations of a dichotomous positive/negative result. [57,58]

Quantification techniques include the standardized uptake value ratio (SUVr), which compares tracer uptake in target versus reference regions and the centiloid scale, which normalizes values based on a scale from 0 (young healthy controls) to 100 (typical AD patients). [58]

To date, three radiotracers have been approved for clinical amyloid PET imaging: [^{18}F] florbetapir, [^{18}F] flutemetamol (FMM) and [^{18}F] florbetaben (FBB), which present different pharmacokinetic proprieties, chemical structure and binding characteristics as summarized in *Table I*.

Guidelines for Performing Imaging and Interpreting Results for the Three FDA-approved Radiopharmaceuticals for Amyloid PET

Radiotracer	Recommended Dose/Activity	Uptake Time (min)	Acquisition Time (min)	Image Display	Number of Regions for Positive Scan
^{18}F-florbetapir	370 MBq (10 mCi)	30–50	10	Gray scale or inverse gray scale	Two, or only one if gray matter uptake exceeds white matter uptake
^{18}F-flutemetamol	185 MBq (5 mCi)	60–120	10–20	Rainbow color scale	One
^{18}F-florbetaben	300 MBq (8.1 mCi)	45–130	15–20	Gray scale or inverse gray scale	One

Sources.—References 18–20.

***Table I**: Guidelines of the three FDA-approved Radiopharmaceuticals for Amyloid PET [56]*

A comparative study suggested that FMM may have superior sensitivity in detecting the amyloid burden in the striatum. However, both FMM and FBB are equally effective in identifying AD pathology in vivo, with no significant difference in cortical SUVr values when referenced to white matter. [59]

In clinical practice, axial PET slices are typically used for primary image reading. The cerebellum is examined first, as it is usually spared from amyloid accumulation in early disease stages and serves as an internal negative control. Coronal and sagittal views are then used to confirm findings and are particularly helpful in evaluating challenging regions such as the precuneus.

Generally, it can be assumed that a PET scan is negative when there is a normal radiotracer uptake in the white matter and no uptake in the grey matter. Conversely, a scan is considered positive when the uptake is observed in both gray and white matter.

Specific patterns of Aβ accumulation in distinct brain regions generate characteristic imaging signs, which can aid in more precise description and localization of amyloid burden (see *Figure 20*). [55]

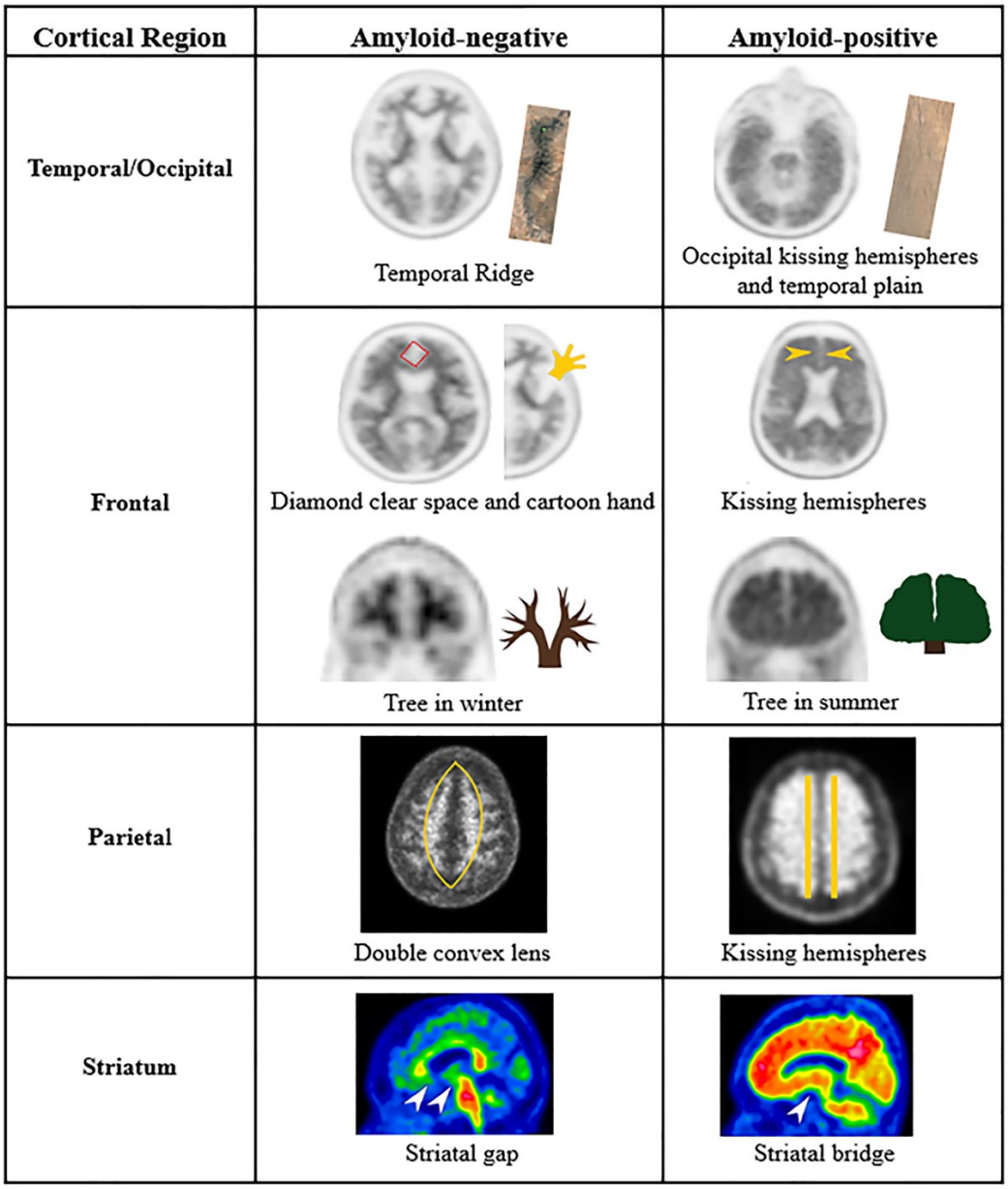

***Figure 20**: Atlas of regional amyloid PET signs [56]*

2.2 DTI-ALPS index and CPL index

Diffusivity was assessed on DWI images at the level of the lateral ventricle body. Spherical ROIs were placed within projection and associative fiber tracts to calculate the DTI-ALPS index. ROIs were transformed from standard space to DWI space through high-resolution T1-weighted images.

The whole brain tractography was generated via anatomically constrained probabilistic tractography implemented in MRtrix3. Information transfer across the structural connectome was quantified using the characteristic path length (CPL) index.

Optimal brain function requires both local specialization and global integration, which are features of a "small world" network architecture, that presents a high clustering coefficient and a short characteristic path length. According to graph theory, such complex systems can be represented by a graph composed of nodes (vertices) and connections (edges). The characteristic path length is defined as the average of the shortest paths connecting all pairs of nodes in the network, while the clustering coefficient quantifies the degree to which nodes in a graph tend to cluster together; it is calculated as the ratio of existing connections between a node's neighbors to the total number of possible connections among them, as illustrated in *Figure 21*.

Recent studies showed that this small world network connectivity may be altered in pathological conditions such as AD [60,61]

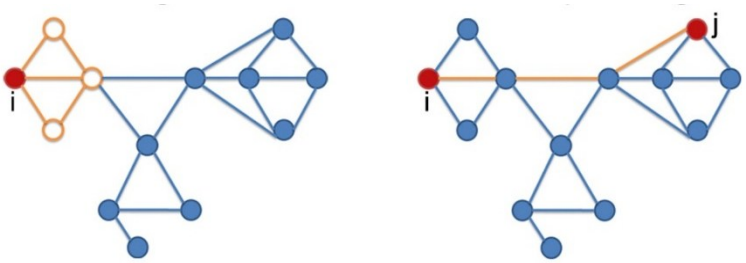

***Figure 21**: Clustering coefficient (left) and shortest path length (right)* [62]

3. Amyloid PET acquisition protocol

Each patient enrolled in the multicenter study was subjected to a PET scan with 18F-FBB or FMM as radiotracer, according to the precise technical instructions provided by the coordinating center (UOC Nuclear Medicine of the University Hospital of Padua).

Regarding the PET acquisition:

- Start the scan (in list mode) before the injection of the tracer, with the patient correctly positioned for brain study and venous access already available;
- After a few seconds, proceed with an intravenous injection of 300 MBq of 18F-Florbetaben, continuing the PET acquisition for the next 15 minutes (0-15 min. Or "Early frame");

- At this stage, acquire also CT with "ultra-low dose" parameters aimed only at correcting the attenuation and not for diagnostic purposes;
- Acquisition pause (the patient returns to the waiting room);
- After about 90 minutes from the injection of the radiopharmaceutical, reposition the patient and perform the late acquisition lasting 20 minutes (90-110 min. Or "late frame");
- At this stage, acquire CT with the usual parameters for brain study.

Regarding PET reconstruction:

- Matrix: 256 × 256 matrix (voxel size 2.32 × 2.32 × 2.03 mm);
- Reconstruction: 3D ordered subsets expectation maximization algorithm;
- Iterations: 8 iterations;
- Subset: 21 subsets;
- Filter: 3-mm Gaussian;
- Corrections: Standard corrections for decay, scatter, dead time and attenuation.

Reconstruct the "Early Frame" PET 0-15 min with the above parameters and with the following times:

- 0-5 min (name the file PET_0_5);
- 0-10 min (name the file PET_0_10);
- 0-15 min (name the file PET_0_15).

Reconstruct the "Late Frame" PET 90-110 min with the above parameters and with the following times:

- 90-100 min (name the file PET_90_100);
- 90-110 min (name the file PET_90_110).

4. Structural and Diffusion MRI Preprocessing Pipeline

T1w preprocessing

Each T1 underwent the following preprocessing steps: modeling and removal of bias-field using N4BiasFieldCorrection as implemented in Advanced Normalization Tools (ANTs), skullstripping using SynthStrip and segmentation

into three tissues via FMRIB Software library (FSL)'s FAST. The segmentation of subcortical gray matter was further refined using FSL's FIRST for model-based segmentation. Each skull-stripped T1w image was then registered to the MNI space using a symmetric non-linear diffeomorphic registration as implemented in ANTs (*SyN* algorithm), preceded by a rigid and an affine transformation for pre-alignment.

DWI preprocessing

Raw DWI data were visually inspected and volumes containing interslice instabilities due to motion were removed. The rest of the preprocessing steps were carried out using the MRtrix3 suite. Firstly, DWI volumes were denoised by exploiting data redundancy via Marchenko-Pastur PCA (Mrtrix3's *dwidenoise*). Then, DWI volumes were simultaneously corrected for B0 inhomogeneity, eddy current and motion using FSL tools as called by MRtrix3's function *dwifslpreproc*. Lastly, volumes underwent N4 bias-field correction. Preprocessed B0 volumes were extracted and averaged to create a B0 template. The average B0 was skull-stripped using *Synthstrip* and rigidly registered to the skull-stripped T1w image (cost function: normalized mutual information) using FSL. The accuracy of each registration was visually checked.

DTI-ALSP index

We downloaded 4 spherical ROIs (diameter: 5mm) corresponding to bilateral superior corona radiata (SCR) and the superior longitudinal fasciculus (SLF) in standard space from (https://github.com/gbarisano/alps/). We inverted the transformations estimated in the T1w and DWI preprocessing pipelines to map the four ROIs first from MNI space to T1w space and then to DWI space. The Diffusion Tensor Imaging (DTI) model was fitted in each brain voxel. We then extracted diffusivities along the x, y and z axes within the ROIs of interest and computed the DTI-ALSP as follows:

$$ALPS = \frac{\mathrm{Dxx, proj} + \mathrm{Dxx, assoc}}{\mathrm{Dyy, proj} + \mathrm{Dzz, assoc}}$$

In brief, DTI-ALPS is computed as the ratio between the mean diffusivity along the x-axis in regions of projection and association fibers to the mean of diffusivity along the y-axis in projection fibers and z-axis in association fibers.

5. Data collection

The processing of personal data was carried out in accordance with current legislation (Code regarding the protection of personal data, Legislative Decree 30 June No. 196/2003 and EU regulation 2016/679). All the data necessary for the research project (visits and images), were anonymized at the satellite Center and subsequently uploaded to an electronic database accessible only to investigators via login and password; each patient was assigned a unique identifier (ID). The PET images obtained were transmitted to the coordinating Center. 15 minutes 18F-Florbetaben scan early phase and 18F-FDG scan were subjected to independent and casual visual assessment by two operators. Eight brain regions were considered for visual assessment: frontal lobe right and left, temporal lobe right and left, parietal lobe right and left, occipital lobe right and left. Signal alteration for each region was estimated by grading scale from 0 to 3 (0-irrelevant, 1-mild, 2-moderate, 3-severe). For each patient MRI T1 sequences were evaluated to identify atrophy and 90-110 minutes late-phase 18F-Florbetaben to detect amyloid deposition.

6. Statistical analysis

Statistical analysis was initially focused on constructing a demographic table for the study population. To do so, a dataset was created using Microsoft Excel, where the mean values and standard deviations (SD) of the variables under investigation were calculated.

To determine the presence of statistically significant differences between groups, independent samples t-tests were employed when comparing two groups. In cases where more than two groups were involved, a one-way analysis of variance (ANOVA) was conducted. Whenever the ANOVA yielded statistically significant results, a post-hoc analysis was performed using Tukey’s Honest Significant Difference (HSD) test, to identify specific group differences.

Descriptive statistics, as well as correlation analyses, were conducted using the Jamovi software (version 2.6.44)**.** Correlation between continuous variables was assessed using either the Pearson correlation coefficient or the Spearman rank correlation coefficient, depending on the data distribution and measurement scale. Specifically, Pearson's correlation was used for continuous variables with normal distribution, while Spearman's correlation was used for ordinal variables or non-normally distributed data.

Confidence intervals and standard errors were calculated where appropriate. Statistical significance was defined as a p-value less than 0.05. All statistical tests were two-tailed.

RESULTS

Demographic and clinical characteristics of the study population

The characteristics of the study population are summarized in *Table II*. Patients were classified into four groups based on disease pathogenesis: amyloid-accumulating (parenchymal or cerebral), synucleinopathies, frontotemporal degeneration (FTD) and other etiologies.

	AD/CAA n = 27	PD/PDD/DLB n = 36	FTD n = 9	OTHER n = 13
SEX	F = 13; M = 15	F = 8; M = 28	F = 3; M = 6	F = 7; M = 6
AGE	65,07 ± 7,27	*71,97 ± 5,67	64,75 ± 6,51	61,31 ± 9,93
MMSE	22,2 ± 4,07	23,58 ± 4,35	20,75 ± 9,05	24,2 ± 6,21
PET AMY	N = 1; P= 26	N = 20; P = 16	N = 13; P= 0	N = 13; P = 0

***Table II**: Characteristics of the study population*

A one-way ANOVA was conducted to compare the mean age across the four groups ($F=9.69$; $p < 0,001$), indicating a statistically significant difference in the mean age ($p<0.05$). A Tukey's HSD post hoc test (see *Table III*) revealed that the mean age of the PD/PDD/DLB group was significantly higher than that of the other groups. The results are summarized in the table below.

Groups compared	Mean difference	p-adj	Lower CI	Upper CI	Significant
AD/CAA vs FTD	0.32	0.900	-5.59	6.22	No
AD/CAA vs OTHER	3.76	0.253	-1.92	9.45	No
AD/CAA vs PD/PDD/DLB	-6.90	*0.001	-11.19	-2.61	Yes
FTD vs OTHER	3.44	0.579	-3.91	10.80	No
FTD vs PD/PDD/DLB	-7.21	*0.008	-12.96	-1.46	Yes
OTHER vs PD/PDD/DLB	-10.65	*0.001	-16.47	-4.83	Yes

***Table III**: Tukey's HSD post hoc test*

Regarding MMSE scores, the ANOVA showed no significant differences between the groups ($F = 1.14$; $p = 0.338$); therefore, no post hoc analyses were performed.

Glymphatic dysfunction and connectivity in AMY+ and AMY- patients

Among patients tested with amyloid PET scans, 57% of AMY+ cases were diagnosed with AD, while the remaining AMY+ patients were primarily diagnosed with other conditions and amyloid pathology was considered a possible comorbidity. This distribution is illustrated in the pie chart in *Figure 22*.

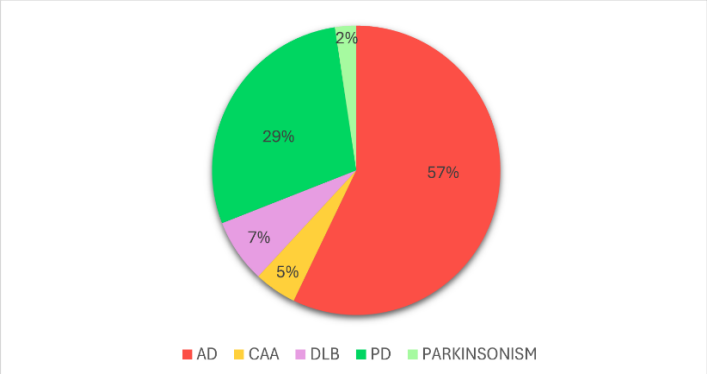

***Figure 22**: Distribution of diseases in the AMY+ group*

Within the AMY- group, the diagnostic distribution differed as illustrated in *Figure 23*.

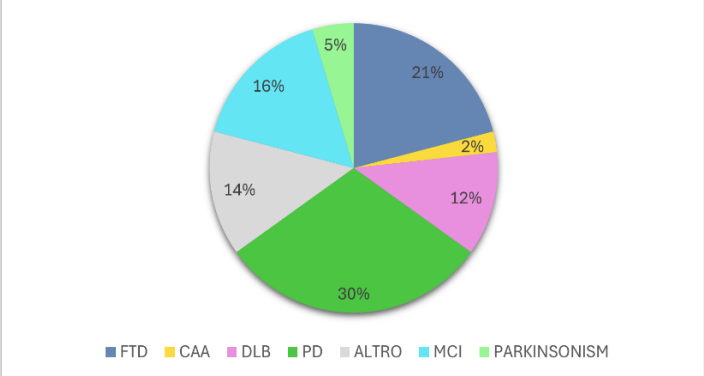

***Figure 23**: Distribution of diseases in the AMY- group*

Statistical analysis comparing glymphatic function in the amyloid-positive and negative group is shown in the violin plot below (*Figure 24*). AMY+ patients had a

significantly reduced diffusivity along perivascular spaces, as indicated by a lower DTI-ALPS index (t = 2.75, p = 0.004), suggesting a reduced glymphatic function.

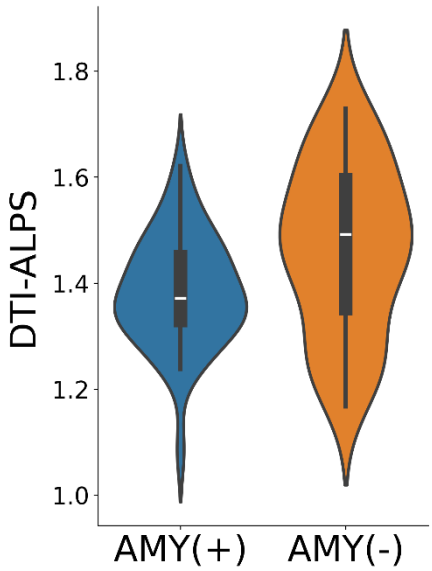

***Figure 24**: Violin plot of the DTI-ALPS index in AMY (+) compared to AMY (-) group*

Furthermore, AMY+ patients demonstrated a significantly higher characteristic path length (CPL) index (t = 2.71, p = 0.006) compared to AMY-, indicating reduced efficiency in information transfer across the structural connectome. (*Figure 25*)

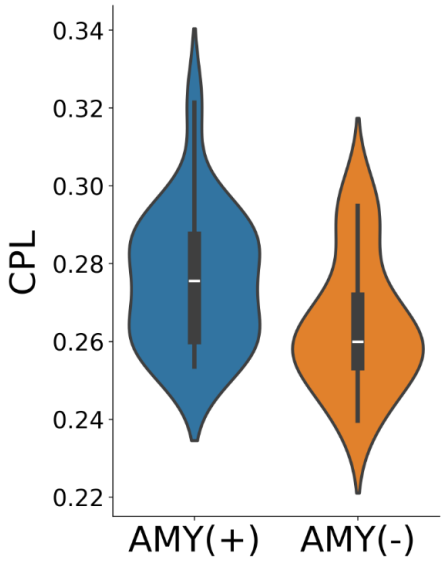

***Figure 25**: Violin plot of the CPL index in AMY (+) compared to AMY (-) group*

The CPL index was negatively correlated with the DTI-ALPS index (Spearman's ρ = -0.37).

Glymphatic dysfunction across diagnostic groups

The box plot in *Figure 26* compares the DTI-ALPS index across different diagnostic groups.

A one-way Welch ANOVA revealed no significant differences in the ALPS index between groups stratified for disease type (F = 1.37, p = 0.286).

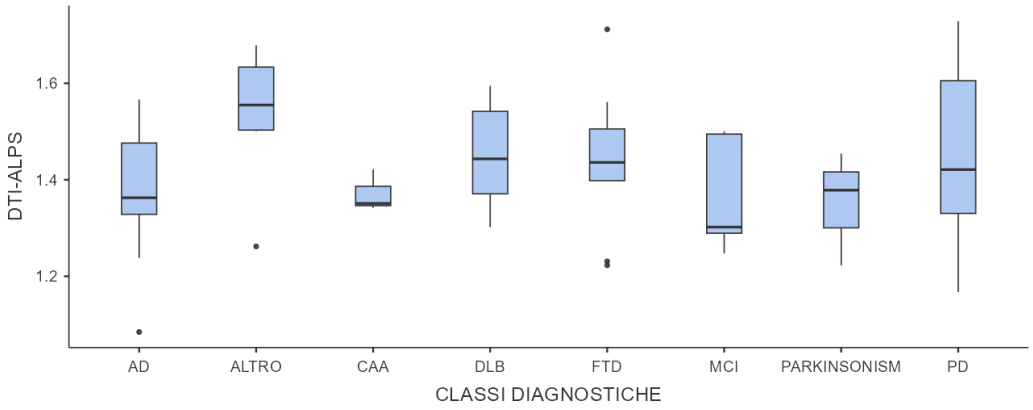

***Figure 26**: Box plot of the ALPS index across different diagnostic groups*

The mean and standard deviation of the DTI-ALPS index for the different groups are summarized in *Table IV.*

	CLASSI DIAGNOSTICHE	DTI-ALPS
Media	AD	1.38
	ALTRO	1.53
	CAA	1.37
	DLB	1.45
	FTD	1.44
	MCI	1.37
	PARKINSONISM	1.35
	PD	1.45
Deviazione standard	AD	0.114
	ALTRO	0.151
	CAA	0.0439
	DLB	0.103
	FTD	0.154
	MCI	0.116
	PARKINSONISM	0.118
	PD	0.163

***Table IV:** Descriptives of the mean and SD of the DTI-ALPS index across different diagnostic groups*

Following classification according to pathogenesis, a one-way Welch ANOVA also found no significant differences in the ALPS index between diagnostic groups (F =

1.48, p = 0.243), even though it is lower in the AD group. Corresponding means and standard deviations are reported in *Table V*.

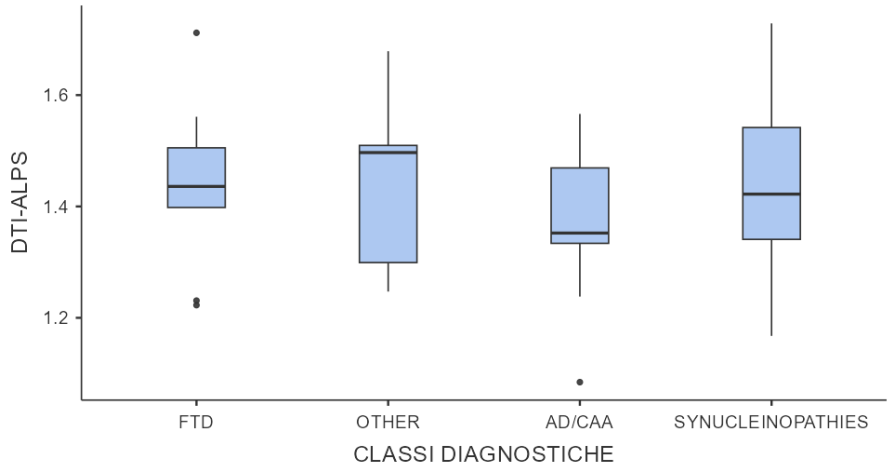

***Figure 27**: Box plot of the ALPS index across different diagnostic group based on the pathological classification*

	CLASSI DIAGNOSTICHE	N	Media	SD	SE
DTI-ALPS	FTD	9	1.44	0.154	0.0512
	OTHER	13	1.45	0.152	0.0420
	AD/CAA	27	1.38	0.108	0.0208
	SYNUCLEINOPATHIES	36	1.44	0.148	0.0247

***Table IV:** Descriptives of the mean and SD of the DTI-ALPS index across different diagnostic groups based on pathological classification*

The dispersion of ALPS index values is further illustrated in the box plot in *Figure 28*, where amyloid-positive cases are marked in yellow and amyloid-negative cases in blue.

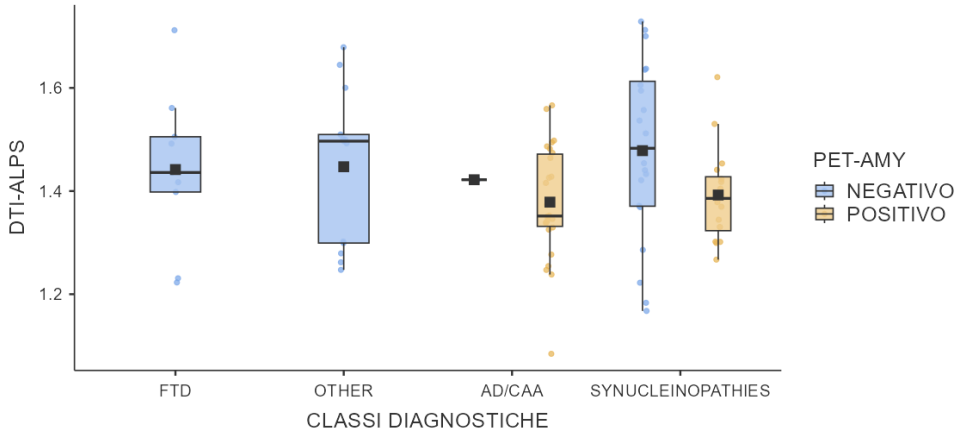

***Figure 28**: Dot plot of the ALPS index across different diagnostic group based on the pathological classification, marking the positive cases in yellow and the negative cases in blue.*

Within the amyloid-positive subgroup, a comparison between AD patients and those with other diagnoses revealed mean ALPS indices of 1.38 ± 0.114 and 1.39 ± 0.087, respectively. A Welch's two-sample t-test (t = 0.32; p = 0.8) showed no significant differences between these groups (Figure 19).

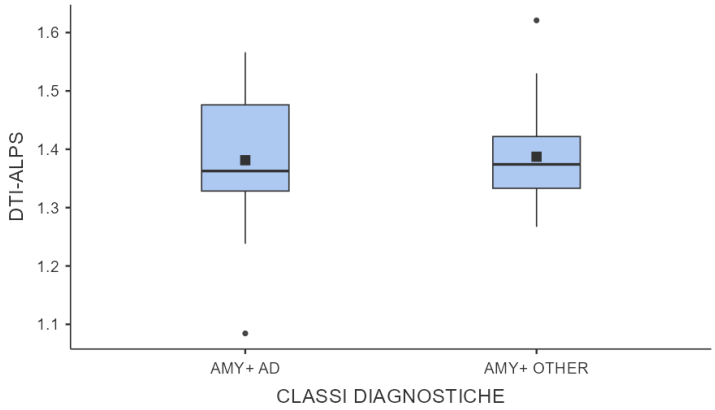

***Figure 29**: Box plot of the ALPS index across AMY (+) patients with AD and AMY (+) patients diagnosed with other dementia*

Correlation between glymphatic dysfunction and clinical severity

The correlation between the ALPS index and MMSE scores was assessed across the entire cohort. Pearson's correlation coefficient indicated no significant association (r = 0.118; p = 0.363). The scatter plot with fitted regression line illustrates the weak or non-significant association.

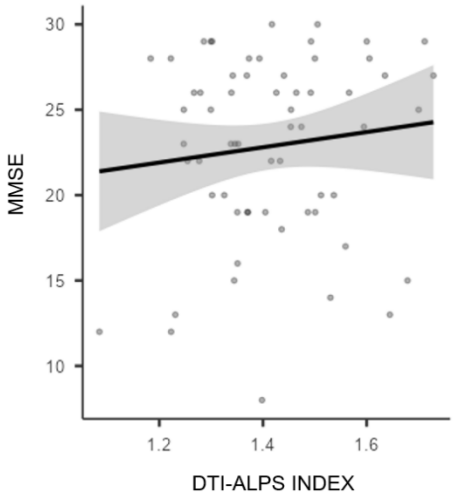

***Figure 30**: Scatter plot with linear regression line; Correlation between ALPS index (x axis) and MMSE score (y axis).*

Separate analyses for amyloid-positive (Pearson's coefficient r= 0,129; p = 0,5) and amyloid-negative group (Pearson's coefficient r = 0,043; p = 0,812) showed a similar non-significant trend, as illustrated in *Figure 31*.

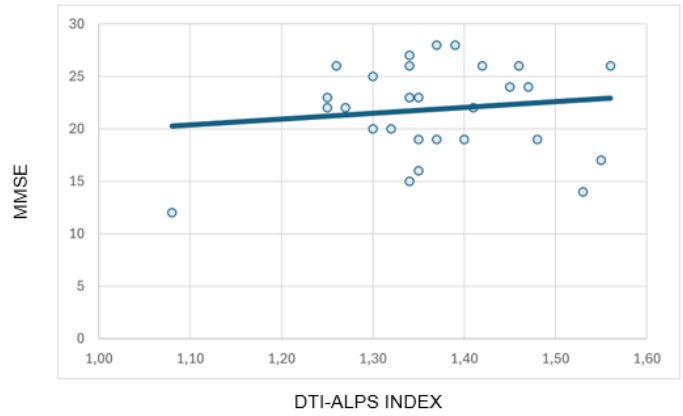

***Figure 31**: Scatter plot with linear regression line; Correlation between ALPS index (x axis) and MMSE score (y axis) in the positive group.*

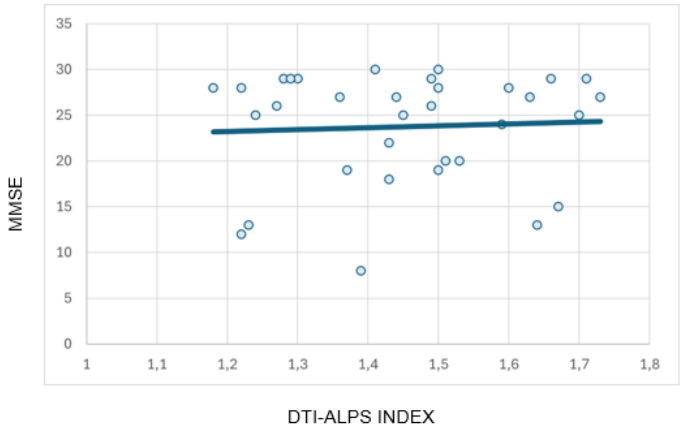

***Figure 32**: Scatter plot with linear regression line: correlation between ALPS index (x axis) and MMSE score (y axis) in the negative group.*

Correlation between glymphatic disfunction and age

The correlation between DTI-ALPS index and age in the entire cohort showed a weak but statistically significant positive association (r = 0.25, p = 0.022). The scatter plot in *Figure 33* illustrates the fitted regression line.

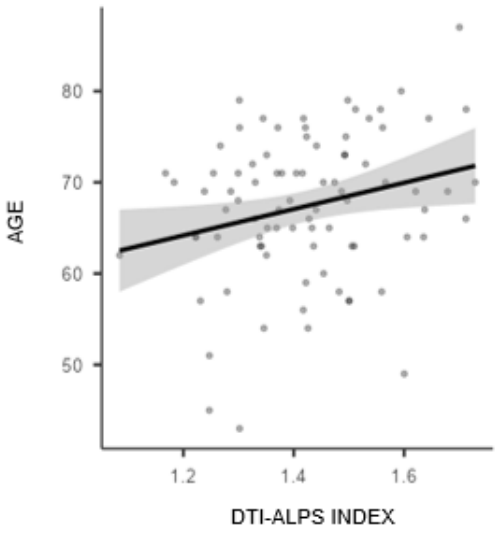

***Figure 33**: Scatter plot with linear regression line: correlation between ALPS index (x axis) and age (y axis).*

When analyzed separately within the amyloid-positive group, the correlation remained positive but lost statistical significance (r = 0.130; p = 0.413) (*Figure 34*).

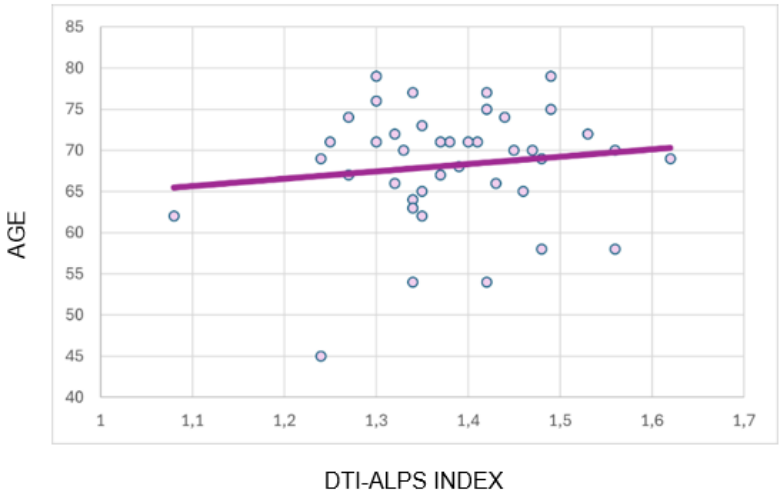

***Figure 34**: Scatter plot with linear regression line; Correlation between ALPS index (x axis) and age (y axis) in the AMY+ group.*

Given that the PD/PDD/DLB group was significantly older than the others, these patients were excluded from the analysis to reduce this potential bias. After

exclusion, the positive correlation persisted but was no longer statistically significant (r = 0.271; p = 0.060) (Figure 35)

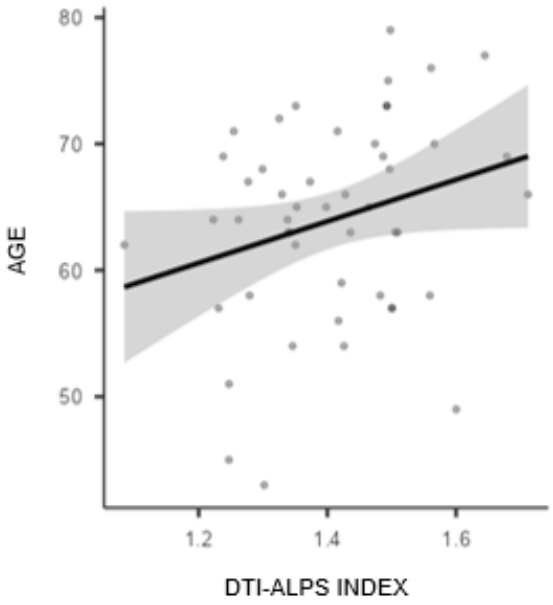

Figure 35*: Scatter plot with linear regression line: correlation between ALPS index (x axis) and age (y axis) after exclusion of the PD/PDD/DLB group*

DISCUSSION

In this study, we explored the glymphatic dysfunction in patients with different types of neurodegenerative dementia, focusing particularly on differences between presence or absence of AD-related pathology, i.e. amyloid parenchymal deposition.

Patients with brain parenchymal amyloid deposition, named amyloid-positive group showed a significantly lower DTI-ALPS index than the amyloid-negative group. Since we used the ALPS index as a proxy for glymphatic function, this finding suggests that the glymphatic system is impaired in individuals with amyloid accumulation, consistent with findings from similar studies. [47,53,62–64]

However, when we compared different diagnostic groups, the differences in the ALPS index were no longer statistically significant.

A possible interpretation of our results is that the amyloid-positive group included not only patients with AD but also those with other neurodegenerative conditions, such as synucleinopathy plus amyloid deposition, presenting amyloid deposition as a comorbidity.

Moreover, it is generally believed that the glymphatic impairment occurs across different types of dementia, not only in amyloid-accumulating cases. As example, some studies suggest an altered glymphatic activity linked to toxic protein accumulation in PD, additionally highlighting the potential of ALPS index changes to indicate the progression from prodromal to clinical PD. [51] However, it is not clear the contribution of PD patients with comorbid AD to these findings, since amyloid markers were not detected. From the results of our study, we can suggest that, at least in part, PD patients can have lower glymphatic function when amyloid co-pathology is associated.

Another study suggested that the glymphatic system is also impaired in genetic forms of FTD and may help predict disease progression across different stages. [65]

Further studies are thus necessary to clarify the association between glymphatic impairment and neurodegeneration.

When comparing the glymphatic function between the amyloid-positive AD cohort and the other amyloid-positive subjects (i.e. PD, PDD, and DLB patients), we did not find significant differences in the ALPS index. This may suggest that

glymphatic dysfunction is primarily linked to amyloid deposition and that the possible concomitant accumulation of other proteins, such as α-synuclein, does not appear to further exacerbate the glymphatic impairment.

Additionally, we calculated the CPL index to investigate the global brain connectivity, finding a significant impairment in patients with a lower ALPS index, indicative of glymphatic dysfunction. This finding suggests that a high amyloid burden, which disrupts glymphatic activity, may also contribute to decrease the global connectivity. This interpretation aligns with the hypothesis that glymphatic dysfunction could disrupt interstitial clearance and connectivity pathways, although further studies are needed to clarify the mechanisms behind this association.

We did not observe a significant association between clinical severity, as assessed by the MMSE scores closest to the PET scan, and glymphatic function. While there was a trend indicating that patients with more severe cognitive impairment had slightly lower glymphatic activity, this did not reach statistical significance.

Prior studies reported mixed findings regarding this association. Zhong *et al.* and *Kamagata et al.* found significant positive correlation between the ALPS index and MMSE and MoCA score; [62,63] while in the work of Sacchi *et al.* the results were comparable to ours. [47] However, it must be considered that Zhong *et al*. and Kamagata *et al*. compared groups with significant differences in clinical severity (AD vs. MCI vs. normal controls), factors that may influence the results. Moreover, significant differences emerged only when compared with healthy controls, whereas — similarly to our findings — the different clinical groups showed similar cognitive performances.

Our findings may be limited by the fact that MMSE scores were not available for all the patients and that the MoCA score, which may provide a more precise assessment of cognitive impairment especially in patients with AD, was not accessible.

Despite these limitations, we believe that the absence of a significant correlation between clinical severity and glymphatic dysfunction is meaningful. It suggests that glymphatic dysfunction may occur early in the disease process, before significant cognitive decline becomes apparent. Consistent with other studies, even when a correlation with neuropsychological tests is found, it is generally not possible to

demonstrate a significant difference in the ALPS index between AD and MCI-AD cases, while a difference is usually observed between AD and normal controls and between MCI and normal controls. [62–64]

The number of MCI (amnestic, non amnestic and subjective) subjects in our cohort was to small to confirm this finding, but the lack of substantial differences among subjects with better cognitive performance seems to align with the results from the other authors.

These findings suggest that glymphatic dysfunction could serve as marker for early diagnosis, since an impairment is already detectable in the MCI stage.

We also investigated the relationship between age and glymphatic dysfunction. Interestingly, we found a positive correlation, with older subjects showing higher ALPS indices compared to younger ones. Since aging is generally considered a risk factor for glymphatic dysfunction, this result may seem counterintuitive. A first possible explanation is that we are not considering healthy aging, but diseases. Therefore, when pathology is present, it seems more severe in young subjects than older ones.

This could be explained by the presence of younger patients with early-onset AD, who may exhibit a higher amyloid burden and consequently a more severe glymphatic dysfunction, reflected by a lower ALPS index. In contrast, older subjects may accumulate amyloid gradually, leading to less pronounced glymphatic dysfunction. This hypothesis could be tested by quantifying the amyloid load and assessing if a correlation with age is present (i.e. younger patients, higher amyloid load).

A possible alternative explanation was that the PD/PDD/DLB group in our cohort was, on average, significantly older than the other groups, potentially skewing the results. For this reason, we excluded these patients and reanalyzed the data. The trend remained the same, but the association was no longer statistically significant. Similarly, when focusing only on the amyloid-positive group, we observed the same trend.

Our study strongly supports the association between amyloid accumulation and lower glymphatic activity. However, some aspects should be further clarified, as the

precise correlation with the amyloid burden, which requires a quantitative assessment rather than a simple positive or negative interpretation. This represents a primary limitation of our study.

Another limitation may be the use of the ALPS index as only proxy of the glymphatic function. This index calculates the diffusion along perivascular spaces in specific anatomical regions to estimate global glymphatic activity and may not represent only the diffusion along the perivascular spaces. On the other hand, multiple studies, including the one performed by Sacchi *et al*, have shown a strong correlation between this index and the glymphatic dysfunction. However, they also suggested that the DTI-ALPS index may be influenced by the degree of structural brain damage and this dependence could limit the index's utility in clinical practice, if used in isolation.

Despite this, we believe that the ALPS index remains a reliable tool to study the glymphatic function, especially when combined with other clinical and imaging markers. Given its non-invasiveness and relative accessibility, we believe that it remains one of the most reasonable and promising proxy for evaluating glymphatic clearance.

To sum up, we trust that our study should encourage further research on this topic to better define the role of glymphatic dysfunction as early diagnostic marker in AD and other neurodegenerative diseases characterized by protein accumulation. Furthermore, additional studies aimed to enhance the glymphatic function as a new therapeutic strategy may offer new and important possibilities in the treatment of neurodegenerative diseases.

In this regard, Mohaupt *et al.* proposed to target the AQP4 expression as promising therapeutic approach, particularly by promoting the expression of the AQP4X isoform, which, based on initial findings, appears to facilitate glymphatic flow due to its higher expression on astrocyte end-feet. [66]

Additionally, other approaches are focusing on brain rhythms as potential targets to reduce the amyloid burden. It has been suggested that multisensory gamma stimulation, by modulating astrocyte end-feet, increasing arterial pulsatility and promoting the release of neuropeptides, may enhance brain clearance. These findings have been observed in AD mouse models, but further studies are needed

to elucidate the mechanisms underlying this potential connection and to assess its therapeutic utility. [67]

CONCLUSIONS

In conclusion we confirmed the results of the current literature regarding the impairment of the glymphatic system in AD and proposed that this is a process mainly guided by amyloid deposition and therefore present also in other neurodegenerative diseases with AD-related co-pathology.

The study of the glymphatic system is therefore crucial for a better understanding of the pathophysiological cascade of AD and potentially other neurodegenerative diseases, opening new possibilities for their treatment.

The interaction between the glymphatic system and neurodegeneration is especially significant, as it indicates that by improving factors such as sleep hygiene and cardiovascular health — both known to disrupt glymphatic function — it may be possible to prevent neurodegeneration.

Additionally, it is essential to further investigate the ALPS index as an early biomarker of AD and to deepen our understanding of its relationship with parenchymal protein accumulation.

Furthermore, the exploration of novel therapeutic strategies targeting the glymphatic system presents a promising approach for addressing neurodegenerative diseases such AD.

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