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PHD COURSE IN NEUROSCIENCE

Curriculum in Clinical and Experimental Neuroscience (XXXV course)

Coordinator: Prof. Lino Nobili

Integrating cerebrospinal fluid and [^{18}F]-fluorodeoxyglucose positron emission tomography to diagnose Alzheimer's disease and research its pathophysiological substrates

Supervisor:

Prof. Matteo Pardini

Candidate:

Dr. Federico Massa

Summary

1. Introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a chronic neurodegenerative disease that is the leading cause of dementia worldwide (Levey 2021). It has a prevalence ranging from 10% to 30% of the population over the age of 65, with a 14-fold increase in over-85-year-old subjects. Sporadic presentation of AD is the most common (>95% of the time) and characterized by a later onset (80-90 years), whereas a little percentage (1%) is caused by gene mutations that affect ß-amyloid transformation, resulting in an earlier onset of disease (median age 45 years) (Masters et al. 2015).

Therefore, AD is one of the main concerns for world health today in the context of the current "aging society". The inexorably expanding incidence and prevalence of this disorder, which has a negative impact on patients, their families, and society as a whole, results in an annual expenditure of around $1 trillion on treatment. As the population over 65 grows, it is anticipated that these costs will continue to rise (Levey 2021).

1.2 Pathophysiology of AD

The fundamental anomaly in AD is the extracellular accumulation of ß-amyloid protein aggregates, which come from the improper processing of amyloid precursor protein (APP), which is crucial for the development and repair of neurons. Normal breakdown of this protein takes place along what is known as the physiological 'nonamyloidogenic route', starting at the extracellular level with a first cut by the enzyme α-secretase and ending with a second cut by γ-secretase at the intramembrane level, yielding soluble amyloid fragments. In the case of AD (amyloid cascade), the first step is carried out by a β-secretase in a different location of the APP, whereas a subsequent γ-secretase-mediated cut results in the production of non-solubilized β-amyloid fragments. These peptides are prone to aggregation as oligomers and neurofibrillary clusters before forming neuritic plaques in the brain parenchyma, triggering an inflammatory cascade driven by microglia and astrocytes. Astrocytic

activation caused by this prolonged process causes reactive gliosis, which worsens synaptic and neuronal damage by producing neuroinflammation and oxidative damage mediators. This state affects how proteins and neurotransmitters move from the soma to the synapse, which is crucial for neuronal function. The disturbance of neuronal cell homeostasis causes the Tau protein, an essential component of the axonal cytoskeleton, to be hyperphosphorylated, which leads to the formation of neurofibrillary tangles (NFTs) consisting of hyperphosphorylated Tau protein. These formations are harmful to cells and enhance synaptic dysfunction and neuronal death farther downstream.

It has been established that the most significant risk factors for sporadic forms of AD are familiarity with the condition, age greater than 65, lifestyle factors such as education and employment, and the presence of the APOE allele epsilon-4 (ε4) located on chromosome 19. Noteworthy, the risk for people who carry ε4 allele in either etero- or omo-zigosis is increased by three to fifteen times, respectively (Masters et al. 2015). It is believed that a potent, dose-dependent influence on the impaired regulation of cerebral amyloid metabolism with aging mediates the elevated APO ε4-related risk for AD. Pathological and amyloid imaging investigations have demonstrated that APO ε4 carriers had higher cerebral amyloid deposition in comparison to non-carriers in both experimental animals and humans. (Morris et al. 2010).

AD physiopathological changes start years before symptoms manifest, although they tend to plateau about the time when people begin to experience symptoms (Jack et al. 2013). According to neuropathological data, amyloid plaques and NFTs have distinct spatiotemporal trajectories of dissemination in the brain. While the latter spreads from transenthorinal and entorhinal regions to the neocortex at various stages, the former starts at the level of the neocortex and then spreads to the allocortex, basal ganglia, midbrain, and finally the pons and cerebellum (Thal et al. 2014). This process affects how the disease develops clinically, revealing a linear correlation between the severity of the illness and the quantity of NFTs and, to a lesser extent, amyloid neuritic plaques (Masters et al. 2015).

Recent research has combined the idea of synaptopathy with the "amyloid cascade" theory, focusing on synaptic damage that would occur before the death of the neuronal body itself. While the specific processes by which amyloid and tau

aggregates harm the synapse are yet poorly characterised, it has been observed that they behave as "neurotoxic species." These proceed to disrupt glutamatergic transmission pathways primarily at the postsynaptic level, specifically by interacting with and overstimulating the NMDA receptor, resulting in excitotoxic calcium influx, oxidative damage, altered axonal transport, disruption of synaptic networks, and ultimately cell death (Li et al. 2018). These mechanisms, which define AD as a proteinopathy and synaptopathy, lead to progressively severe parenchymal atrophy that is accompanied by cognitive impairment until overt dementia.

1.3 Clinical course

An increasingly incapacitating loss of cognitive abilities, beginning with episodic, semantic, and visuospatial memory and progressing to semantic fluency, praxis, and executive skills, is a characteristic feature of AD. Behavioral and psychological symptoms of dementia (BPSD), which inevitably accompany the clinical progression, frequently also manifest from the outset, and include symptoms such as apathy and depression, anorexia, anxiety, and agitation.

Mild cognitive impairment (MCI) characterizes the early stages of AD and is a condition that resides in a "grey area" between a functional state that is preserved and decline into full-blown dementia. MCI is characterized by a broadly defined clinic, a subtle issue in one or more cognitive domains, objectified by neuropsychological testing, and differs from dementia in that everyday activities are unchanged (Petersen 2004). As reviewed by Roberts and Knopman (2013), approximately 15 to 20 percent of adults over 65 have MCI, and those with MCI and associated memory deficits are more likely to develop AD (or other dementias) than those without MCI. The cohorts analyzed show that transition to dementia occurs in occurs in more than 50% of individual with MCI, with an annual conversion rate varying from 4 to 31.1% (Bruscoli and Lovestone 2004).

1.4 Biomarkers of AD pathology

To examine the probability that the clinical state of MCI represents an early stage in the spectrum of AD (or other neurodegenerative disorders) that may later develop to dementia with a different likelihood, it is imperative to discover measurements that can be applied in the diagnostic process. This is consistent with how recently developed academic and clinical perspectives on neurodegenerative illnesses. In fact, the traditional diagnostic methods based only on clinical presentation, and eventually confirmed by post-mortem neuropathological findings, has been replaced by a biomarker-based approach involving different biological measures which can reflect pathological changes *in vivo*. By definition, biomarkers are measurable indicators of some biological state or condition allowing in-depth understanding of molecular aberrations and physiopathological processes concurring in neurodegenerative conditions, thus guiding clinicians in the differential diagnostic pathway and researchers in the development of new therapies.

Particularly significant for the AD condition are morphologic, molecular imaging and cerebrospinal fluid (CSF) biomarkers that enable capturing the three primary pathophysiological processes, namely amyloidosis, tauopathy, and neurodegeneration, which are outlined in the AT(N) framework (Jack et al. 2018) that will be thoroughly discussed in the paragraph 1.5.

1.4.1 Morphologic imaging

Magnetic resonance imaging (MRI) can enable for estimation of the regional, structural integrity and volume loss and the detection of other brain disorders, such as space-occupying or vascular lesions, which may present with a cognitive impairment (Frisoni et al. 2010). Several studies have found that structural MRI estimations of tissue damage or loss in typically sensitive brain areas such as the hippocampus and entorhinal cortex are predictive of MCI progression to AD and can be quantified through visual metrics, such as the Medial Temporal Atrophy (MTA) scale (Scheltens et al. 1992) (Figure 1).

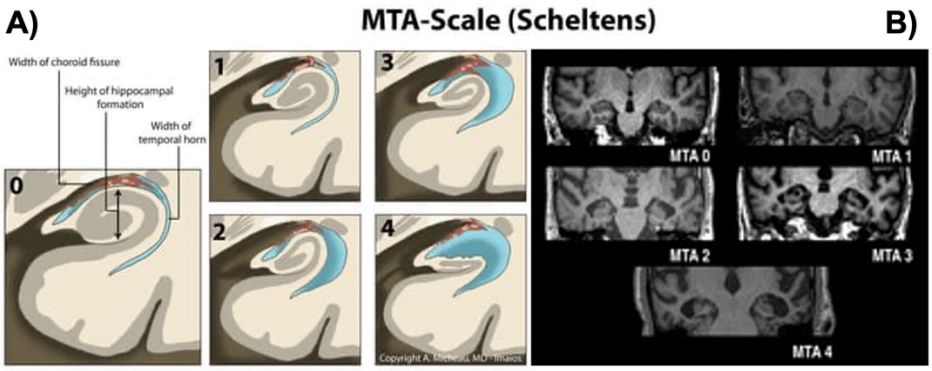

Figure 1. Graphical representation (A) and coronal T1-MRI sequence (B) for the assessment of mesial temporal lobe atrophy (Scheltens et al. 1992). The score value is determined by the size of the choroid fissure, the enlargement of the temporal horn, and the height of the hippocampal formation, as follows 0: no CSF is visible around the hippocampus; 1: choroid fissure is slightly widened; 2: moderate widening of the choroid fissure, mild enlargement of the temporal horn and mild loss of hippocampal height; 3: marked widening of the choroid fissure, moderate enlargement of the temporal horn, and moderate loss of hippocampal height; 4: marked widening of the choroid fissure, marked enlargement of the temporal horn, and the hippocampus is markedly atrophied and internal structure is lost

1.4.2 Cerebrospinal fluid biomarkers

Alzheimer's disease (AD) may be accurately diagnosed with the use of cerebrospinal fluid (CSF) biomarker analysis, which further enables to thoroughly examine the pathophysiological mechanisms underlying each stage of these diseases. The analysis of CSF following lumbar sampling (L3-L4/L4-L5) is able to quantify the concentration reflecting the rates of both production (expressed, released, or secreted by neurons or glia) and clearance (degraded, removed) of specific proteins at the moment of sample, providing the *biological fingerprints* of AD.

With regard to the diagnosis of AD, the core CSF biomarkers specifically represent amyloid pathogenesis, leading to tauopathy (i.e., formation of the neurofibrillary tangles, NFT) and neurodegeneration according to the "amyloid cascade" theoretical model. These proteins include:

- Amyloid-1-42, alone or normalized based on the concentration of the Aβ40 isoform (Aβ42/40 ratio), which reduce in both cases at the CSF level by increased parenchymal sequestration and decreased clearance as an index of amyloid deposition in the brain parenchyma;
- Tau protein phosphorylated at threonine-181 (p-Tau), which represents NFT progressive formation and rises in CSF levels in response;

- total Tau protein (t-Tau), which increases in CSF in parallel to neuronal injury.

In addition to the proteins described above, the CSF level of proteins expressing numerous pathophysiological processes seen in AD, such as neuroinflammation, axonal and barrier/pericyte damage, axonal damage, and synaptic degeneration, can be measured. The latter will be covered in greater depth later.

1.4.3 Positron Emission Tomography

Positron Emission Tomography, or PET, is a technique that uses organic compounds labelled with positron-emitting radioisotopes that enable to examine the regional glucose metabolism ([18F]-FDG PET), or the presence of pathological aggregates of proteins of interest. As for the latter, amyloid-PET will be discussed in the following paragraph due to its widespread usage and the substantial evidence supporting its diagnostic performance will be covered in the next paragraphs, whereas Tau-PET, which allows for detection of brain aggregates of Tau protein, still requires full validation and is still mostly available at a few research institutions.

1.4.3.1.1 Amyloid-PET

The produced fluorinated tracers [^{18}F]-florbetaben, [^{18}F]-florbetapir, and [^{18}F]-flutemetamol) are highly selective for binding amyloid, but due of their lipophilicity, they are also diffusely and powerfully taken up at the level of the white myelin material. They lack horizontal specificity, failing to distinguish between amyloid in its various conformations, such as neuritic plaques (typical of AD and neurodegenerative diseases with cerebral amyloidosis), diffuse extracellular plaques (more typical of senile age), and amyloid angiopathy, but they do not bind other pathological proteins, such as tau and alpha-synuclein. AD, including prodromal forms (MCI-AD), and a significant fraction of individuals with dementia with Lewy bodies (DLB), in whom amyloidosis is linked with alpha-synuclein deposits, are all known to disclose significantly increased retention of amyloid tracers in the brain. Overall, the results for the three fluorinated tracers and for both visual and quantitative analyses ranged from 89% to 97% for sensitivity to distinguish AD patients from healthy people, but the values for specificity varied more significantly, from 63% to 93% (Morris et al. 2016). Indeed, a variable proportion of the aged population has been shown to have cerebral

amyloidosis (10-15% at age 60, 30% at age 75, and higher after age 75), with a link to the Apo ε4+ genotype (where 50% positive is attained at age 75 among cognitively healthy patients). Therefore, it is essential to understand the meaning of a positive amyloid PET scan, indicating the existence of cerebral amyloidosis that allow accomplishing the 'A' criteria of the AT(N) framework for the Alzheimer's continuum condition (Jack et al. 2018), but can be prone to false positives in the elderly.

1.4.3.1.2 [^{18}F]-FDG-PET

The ability to quantify the rate of glucose consumption in each region of the brain gives [^{18}F]-FDG PET its distinctive quality, providing information on the distribution of synaptic dysfunction and *in vivo* neuronal death. Since [^{18}F]-fluorodeoxyglucose (FDG) is a glucose analog, it is readily taken up by the cells with the highest metabolic activity, such as brain cells, where it is phosphorylated and rendered inert. The presence of fluorine-18 ([^{18}F]), a radioisotope that may generate positrons that can be detected by the PET tomograph, results in a steric clutter that prohibits [^{18}F]-FDG from undergoing glycolysis, unlike glucose. As a result, the molecule emits a signal that is "visible" to the tomograph for as long as it is radioactive and confined inside the cell. By analyzing the distribution of the tracer in various brain regions, it is possible to identify patterns that might aid in making the right diagnosis.

As a general interpretation, the region is less intact and synaptically functioning the less metabolically active (and so hypocaptant) it is, and vice versa. Because the metabolic consumption of glucose specifically takes place at the level of the neuron-astrocyte synaptic unit (neuron-glia metabolic coupling) (Magistretti 2000) hypometabolism in a given brain region is likely to be associated with lower synaptic density or neuronal suffering, or it could be an expression of deafferentation from another functionally-connected pathological region (diaschisis phenomenon), even before presenting neuronal damage. This latter point explains why [^{18}F]-FDG-PET has higher sensitivity as a biomarker of neurodegeneration than traditional morphological imaging (MRI), particularly in the early stages of a neurodegenerative process, and a resulting significant negative predictive value (Nobili et al. 2018a). More particular patterns in the setting of dementia, and notably in AD, may be found exactly by studying the distribution of metabolism. In terms of AD, the temporo-parietal regions,

which comprise the angular gyrus, precuneus (PC), and posterior cingulate (PCC), are known to be the most often hypometabolic areas (Bohnen et al. 2012). Although asymmetrical, the basal hypometabolism detected is often bilateral. Conversely, it is typically challenging to detect frank hypometabolism at the mesial temporal level (entorhinal cortex, including the hippocampus, and amygdala), despite the fact that this area is primarily affected by the neurodegenerative process, as there is physiologically lower basal metabolism in these areas and the volume of these structures also varies across subjects. Therefore, it is thought that the most accurate and early indicator of AD is the hypometabolism of the posterior cingulate (PCC), which is frequently extended to the precuneus (PC) (Morbelli et al. 2015a). This pattern involving PC/PCC hypometabolism, which expresses a functional disconnect with the hippocampus, is helpful in the differential diagnosis of pathologies like dementia with Lewy bodies (DLB), which typically presents with occipital hypometabolism but with relative sparing of the posterior cingulate ("cingulate island sign") due to less hippocampal involvement (McKeith et al. 2017) (Figure 2).

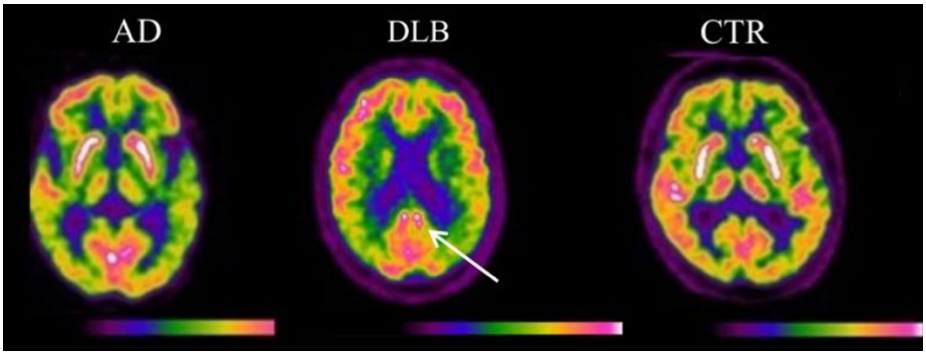

Figure 2. [^{18}F]-FDG-PET metabolic patterns of AD with the predominant involvement of the temporo-parietal cortex, posterior cingulate and precuneus of the classic form (AD); parietoccipital hypometabolism with relative sparing of the posterior cingulate, (cingulate island sign, CIS, white arrow) in dementia with Lewy bodies (DLB); normal metabolism of the healthy subject (CTR)

Automated semi-quantification methods can be used in conjunction with conventional visual analysis of FDG-PET images to increase diagnostic accuracy and reduce the weight of readers' expertise (Nobili et al. 2018b), especially in the early phases of cognitive loss, when there may only be minor changes in brain metabolism (Morbelli et al. 2015a; Massa et al. 2021). Among the many tools, there are specific software that differ primarily in the reference region used for normalization, analysis

methodologies (voxel-based, VBA, or volume-of-interest, VOI-based), the composition of the control group, and the selection of statistical significance threshold.

For instance, each PET voxel can be compared between an individual (single-subject analysis) or a group of subjects (group-analysis) and one or more control groups using a voxel-based analysis (VBA). This results in a parametric map that shows the voxels where the relative decrease in glucose metabolism is statistically significant (based on a pre-set threshold) (Perani et al. 2014). By defining VOIs (volumes of interest), this evaluation may also be applied to clusters made up of statistically significant groups of adjacent voxels. From these, mean metabolic density counts that are similar between groups can be calculated, adjusted to whole brain (WB) metabolism (VOI-based analysis). The semi-quantification technique permits correlation analyses between metabolism and other quantitative variables of interest, such as demographic data, neuropsychological scores, or biological indices.

1.5 A novel biological definition of AD: the AT(N) classification

Through the AT(N) framework, the National Institute on Aging and Alzheimer's Association (NIA-AA) recommended updated research standards for AD, incorporating the idea of a "research framework" within the context of the biological development of the illness (AD-continuum) (Jack et al. 2018). This method shifts the emphasis from a solely clinical (signs/symptoms) and cognitive focus to the pathophysiology underpinnings of the illness. As a result, the new framework is based on the investigation of particular biomarkers that attest to the presence of toxic proteins and neurodegeneration for a definition of the disease that becomes biologically based. Therefore, the AT(N) approach enables classifying individuals based on their biomarker profiles outside of the clinic and using a common language with which researchers can comprehend and characterize a precise etiopathological sequence underlying the AD continuum.

Specifically, the use of biomarkers allows definition of:

- amyloidosis (A), namely extracellular amyloid deposits, that is indicated by either reduced Aβ42 levels, alone or normalized on the value of the Aβ40 isoform (Aβ42/40 ratio, or abnormal PET imaging with tracers for amyloid plaques;

- tauopathy (T), namely the presence of aggregates of hyperphosphorylated Tau protein (neurofibrillary tangles, NFT) connected to elevated CSF p-Tau or abnormal PET imaging with tracers for Tau protein;
- neurodegeneration or neuronal injury (N) that can be shown by volume loss at morphological imaging, decreased regional brain metabolism in [^{18}F]-FDG-PET scan, or elevated t-Tau concentration in the CSF.

Precisely, the presence of amyloidosis determines if an individual is on the Alzheimer's continuum, whereas additional tauopathy whether an individual on the Alzheimer's continuum respects the pathophysiological requirements for AD., Neurodegeneration, on the other hand, is a non-specific process that may occur in a variety of diseases, and can be employed as a proxy for severity in AD staging (Figure 3).

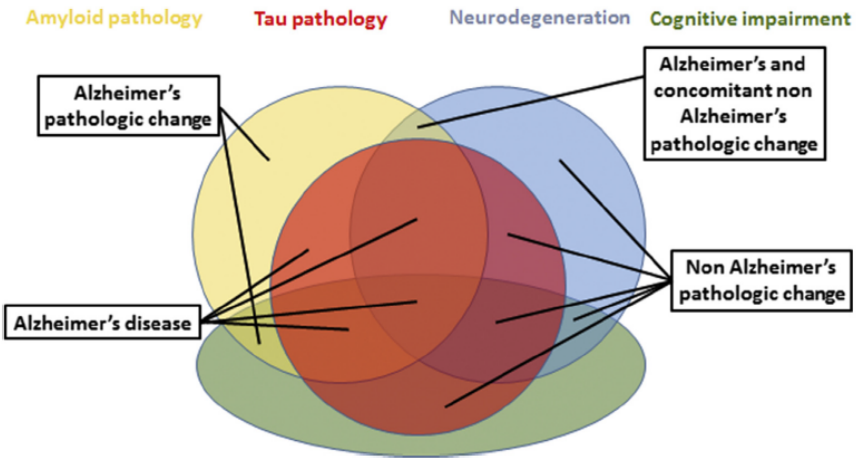

Figure 3 depicts the interaction of AT(N) system categorization and cognitive state from Jack et al. (2018): Alzheimer's pathologic change (A+, in yellow); substantial biomarker alterations suggestive for Alzheimer's disease (A+T+, A+T+(N±)), in red; no evidence of amyloidosis, hence non-AD condition (A-T±(N±), in blue).

1.6 Expanding the AT(N) system: focus on synaptopathy in AD

The AT(N) biomarker matrix theoretically might serve as the foundation for the creation of a larger system, ATX(N), where X denotes new potential biomarkers for other processes that contribute to the pathophysiology of AD, such as changes in the blood-brain barrier, synaptic dysfunction, and neuroimmune dysregulation. For example, vascular, inflammatory or synaptic biomarkers could be integrated to create ATV(N), ATI(N) or ATS(N) systems, respectively (Hampel et al. 2021).

Although the precise mechanisms are still uncertain, evidence suggests that a decrease in synaptic activity and density is one of the first events that would occur before neuronal death in a variety of CNS disorders, including Alzheimer's, which can therefore be classified as "synaptopathies" (Masliah et al. 2001; Taoufik et al. 2018). In AD, synaptic degradation outweighs and precedes neuronal loss at the cortical level and has stronger impact on cognitive symptoms than amyloid plaques or neurofibrillary tangles (Blennow et al. 1996). The amyloid cascade and subsequent buildup of oligomers that obstruct vesicular transport at the synapse level would be the mechanisms that lead to synaptic loss in AD. This multi-step process starts with a reversible initial phase that involves excitatory synapses and is characterized by dysregulation of NMDA and AMPA glutamatergic receptors and those membrane proteins ensuring proper positioning of these channels at the synaptic membrane. It may be assumed that the synapse and its network would already be dysfunctional in the early degenerative phases of disease, but as the synapse gets increasingly damaged, the pre-synaptic terminal and dendritic spines (post-synaptic side) really begin to deteriorate. According to some research, enhanced synaptic sprouting may be an initial protective mechanism, which is then followed by axonal degeneration and ultimately neuronal death in the pathology's later stages (Berchtold et al. 2013; Overk and Masliah 2014) (Figure 4).

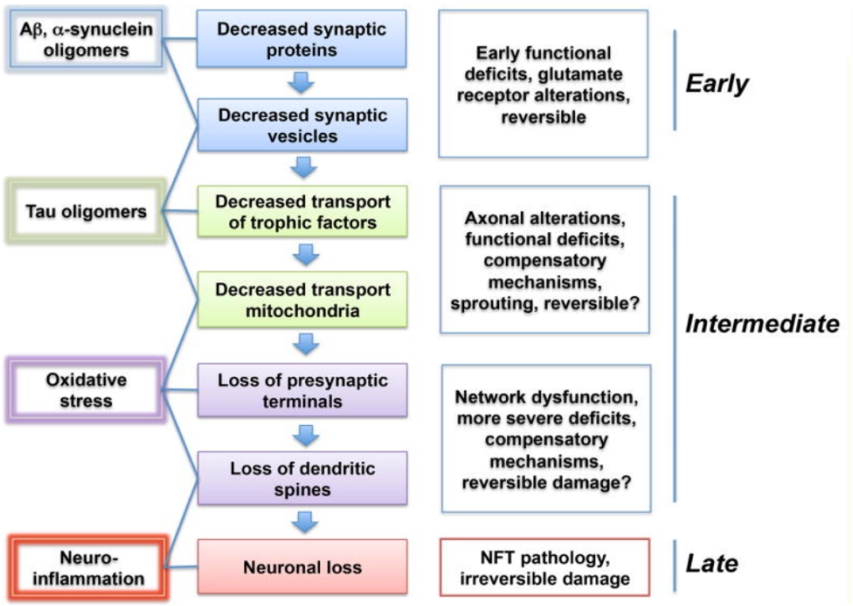

Figure 4. Diagram of the evolution of the synaptic damage process in AD (Overk and Masliah 2014)

It has been postulated that altering synaptic plasticity is a critical component in the pathophysiology of AD. A significant component would be the neuron's aberrant self-organization, which arises from the dynamic process of compensation and then functional decompensation of the synapse (Arendt 2009). Furthermore, as a site of signal transmission between neurons, synapses may contribute to the spread of disease through a "prion-like" process (Guo and Lee 2014), permitting transmission of tau- and amyloid-oligomer aggregates from one neuron to the next.

In an effort to characterize synaptic dysfunction *in vivo*, several pre- and post-synaptic proteins were described in the last years, but evidence of their involvement in the pathophysiology of AD is sparse (Camporesi et al. 2020). Among them, neurogranin (Ng), a dendritic protein revealing post-synaptic integrity, and two pre-synaptic proteins of the family of synucleins (beta-synuclein, β-syn, and α-synuclein, α-syn) increase in the CSF of AD patients along with cognitive impairment and disease progression (Wellington et al. 2016; Oeckl et al. 2016, 2020; Monge-Argilés et al. 2020; Halbgebauer et al. 2021). As the focus of the study project, these proteins will be explained in more depth.

Neurogranin (Ng) is a neuron-specific postsynaptic protein involved in memory consolidation processes and long-term potentiation (LTP) circuits. It is highly expressed by excitatory neurons in the cerebral cortex, hippocampus, and amygdala at the cell body and dendritic spines levels that are involved in calmodulin regulation and commonly affected in AD. Hence, Ng it is seen as a potential marker of synapsis loss in the AD context (Kester et al. 2015; Blennow and Zetterberg 2018). Ng levels in the CSF have been shown to be much higher in AD patients than in those with other types of dementia as early as the MCI stage and to have a strong correlation with cognitive decline and the rate of progression to dementia. Furthermore, in individuals with MCI, the CSF concentration of Ng was found to be directly related to that of t-Tau and p-Tau and indirectly to that of A42, indicating how neurogranin is a sensitive marker for AD alterations (Kvartsberg et al. 2015; Wellington et al. 2016; Tarawneh et al. 2016; Wang 2019).

α-synuclein (α-syn) is a protein predominantly expressed at the level of the pre-synaptic termination and marginally in the nuclear membrane of neurons in the cerebral cortex, cerebellum, striatum, thalamus, hippocampus, and olfactory bulb.

Alterations α-syn often take place in the pathogenesis of AD despite typically being connected to synucleinopathies, i.e., Parkinson's disease, dementia with Lewy bodies and and multisystem atrophy It has been shown through research on murine and human cell cultures that α-syn is involved in the phosphorylation processes of tau protein (mediated by the GSK3 system), which would explain the strong correlation between α-syn and tau levels in CSF found in several neurodegenerative diseases, including AD. Furthermore, it appears that the presence of amyloid beta enhances GSK3 activity, increasing tau phosphorylation and α-syn synthesis. α-syn, tau, and amyloid complexes would encourage mutual aggregation, which might enhance cellular malfunction, and ultimately death (Twohig and Nielsen 2019). Additionally, several studies have shown a connection between cognitive impairment and high CSF levels of α-syn, which are also connected to amyloid deposition in those patients at risk for sporadic AD and familial AD (Berge et al. 2016; Twohig and Nielsen 2019).

Another presynaptic protein that is a member of the synuclein family and that is mostly expressed in the striatum, thalamus, neocortex, cerebellum, and hippocampus is known as β-synuclein (β-syn). It is mostly concentrated in the pre-synaptic terminal, where it plays a part in a number of membrane activities (Barba et al. 2022), and during synaptic degeneration it is released into the synaptic space, the extracellular area and subsequently the CSF. In fact, CSF levels of β-syn were shown to be greater in the in patients with AD at either MCI or dementia stage compared to other neurodegenerative conditions, indicating that this protein represents a promising biomarker of synaptic damage in AD (Oeckl et al. 2016; Halbgebauer et al. 2021). More recently, we showed that CSF β-syn increases in the whole AD continuum since the preclinical stage and may be a helpful early diagnostic/prognostic marker (Barba et al. 2023).

1.7 Issues with the use of diagnostic biomarkers in Alzheimer's disease

The aforementioned biomarkers of AD are spread among centers dedicated to cognitive disorders and dementias, and several data are available about their specific features and accuracy (see for a review Fiandaca et al. 2014). Although, pushed by clinical needs, scientific and technical advances and commercial opportunities, the widespread use of diagnostic biomarkers has actually overtaken both the standardization and harmonization of the various methods and the definition and

validation of targeted diagnostic pathways by directly comparing performances among the various biomarkers (Frisoni et al. 2017).

Several initiatives promoted by the pertinent international scientific societies tried to address issues related to biomarker standardization and clinical application, mainly focusing on one biomarker or class of biomarkers, often without a common synergic framework which could have ensured a global consistency. As for an example, the CSF biomarkers for AD are at an advanced stage of development and harmonization; though, the manual enzyme immunoassays that have been used in most laboratories worldwide are sufficiently reliable and accurate only when used by an experienced staff with quality control procedures. A potentially important advancement is the development of ultra-sensitive, fully automated assays (Bittner et al. 2016; Mattsson-Carlgren et al. 2022) able to reduce the analytic variability. Moreover, efforts are focused on standardization of the operative protocols for CSF handling to reduce pre-analytical variability due to contamination, collection, and storage of samples with the aim to facilitate comparison of CSF biomarkers across studies and laboratories (Hansson et al., 2018). Consequently, a highest performance and reliability of CSF biomarkers analysis, by eliminating most confounding variables, may allow more accurate comparisons among different biomarkers in the diagnostic and prognostic evaluation of patients.

Furthermore, research on the most cost-effective and reliable flowchart for directing biomarker use is currently lacking. The studies comparing biomarker performance do not conclusively show that one is superior than the others (Herukka et al. 2017; Simonsen et al. 2017). As an example, a trend in favor of a higher accuracy of FDG-PET than CSF biomarkers reported by some papers (Shaffer et al. 2013; Prestia et al. 2015; Caminiti et al. 2018) has not been confirmed by a meta-analysis with pathological confirmation, reporting instead a similar accuracy (Cure et al. 2014). Several studies have indicated that combining several indicators enhances diagnostic value to using just one (Bouwman et al. 2007; Vemuri et al. 2009; Choo et al. 2013; Prestia et al. 2013). However, conducting all (or most) biomarkers in an MCI patient is not cost-effective because the majority of patients may be identified appropriately with a single biomarker and only those with ambiguous findings may have access to a

second examination. In this case, a progressive strategy might lead to a cost-effective and precise diagnosis.

There is considerable discussion over which biomarker should emerge first and for which clinical presentation. Both CSF biomarkers and FDG-PET have good diagnostic accuracy and are suggested as first-line biomarkers in MCI patients by scientific organizations (Herukka et al. 2017; Arbizu et al. 2018; Nobili et al. 2018a). When CSF biomarkers are available, they are increasingly recommended if the primary clinical suspicion is AD, but FDG-PET is recommended if alternative diagnoses are at least as likely as AD (Boccardi et al. 2019; Chételat et al. 2019).

Awaiting forthcoming internationally agreed-upon and evidence-based proposals (Festari et al. 2022), even in the clinical routine of centers with high-level of expertise in the management of neurodegenerative diseases, the choice of diagnostic biomarkers often relies on the confidence of the clinician, local facilities, waiting lists, cost, expertise and performance of local laboratories, rather than on the specific characteristics of the biomarker itself and on evidence-based considerations (Frisoni et al. 2017). Thus, to define an efficient combination of biomarkers in AD there an urgent need to accumulate evidence on their analytical and diagnostic performance, when used alone, simultaneously or in a step-wise fashion. This is intended to assess the discriminant accuracy and the added role of one over another in the diagnostic flowchart, and complements the effort in validating and sharing accurate standardized operative procedures.

2. Thesis aims and outline

We have emphasized how various biomarkers are now essential to the diagnostic and research route in the context of research increasingly focusing on the series of pathophysiological events that characterize the course of AD.
In this research project, I have focused on two different types of biomarkers, namely CSF and [^{18}F]-FDG-PET, that are each unique to different pathophysiological processes but that can be integrated to yield complementary data.

Specifically, my project intended to add to the body of knowledge regarding the diagnostic accuracy of biomarkers in AD and to expand the current understanding of some particular pathophysiological aberrations, including synaptic dysfunction.

The first study below (**Chapter 3**) is part of the efforts directed toward defining a diagnostic tree that considers the main characteristics of each biomarker, either alone or in sequential combination, and a cost-effective approach to identifying patients in whom the addition of a second biomarker might be truly useful. This addresses the need to find the best method to achieve the highest diagnostic accuracy using the fewest biomarkers for clinicians as well as for national health care systems, as the number of people with MCI is anticipated to gradually increase, along with the costs provided by diagnostic procedures, care, and hopefully forthcoming disease-modifying drugs. Precisely, we assessed the reciprocal incremental diagnostic value of CSF biomarkers *versus* [^{18}F]-FDG-PET and *vice versa*, utilized one after the other, in individuals with MCI whose first biomarker result had been inconclusive in making the diagnosis. In order to achieve this, we looked on retrospective data collected for clinical purposes from two centers that specialize in the diagnosis and treatment of neurodegenerative disorders. According to clinical practice, either CSF biomarker assessment was used first, followed by [^{18}F]-FDG-PET if this was insufficient for diagnosis, or *vice versa*. We first reported whether the second biomarker utilized in these two cohorts resulted in a diagnosis, and then we evaluated how many of these diagnoses were confirmed at longitudinal follow-up, which was regarded as the gold standard for confirming diagnoses. Finally, we could calculate an incremental relative diagnostic value of 30.6 percent for CSF compared with an inconclusive [^{18}F]-FDG-

PET and vice versa of 38.5 percent. Therefore, we discovered a slight advantage for FDG-PET over CSF in making the most accurate diagnosis; consequently, in practical terms, we might suggest FDG-PET when the diagnostic scenario has not been narrowed by first-line assessments and still includes other diseases besides AD. On the other hand, adding CSF might be useful when we need a specific confirm of a valid suspicion of AD, which FGD-PET initially was unable to resolve.

In the second study of my research project (**Chapter 4)**, we have leveraged the intrinsic abilities of [^{18}F]-FDG-PET as a multifunctional measure of neuron-astrocyte metabolic unit activity, neuropil loss, and synaptic density, which is sensitive to the phenomenon of deafferentation (diaschisis) and axonal degeneration with neural network degradation (Herholz 2003; Nakashima et al. 2007; Zimmer et al. 2017; Provost et al. 2021). Through the course of AD, these mechanisms shift in relative importance, which affects the topography and degree of hypometabolism in individuals and stages differently (Brown et al., 2014; Mosconi, 2005; Silverman al., 2001). According to this concept, specific topographic correlations of metabolic values with CSF proteins that represent distinctive underlying disease processes can be hypothesized. For this reason, we concentrated on a group of 26 patients with MCI-AD to investigate the brain metabolic correlates of certain newly discovered CSF synaptic biomarkers (Ng, α-syn, β-syn) through the semiquantitative analysis of [^{18}F]-FDG-PET scans. We additional extended the analysis to the neurofilament light chain (NfL), whose level in CSF rises in AD as early as the MCI stage in correlation with cognition, amyloid load, and cortical atrophy (Zetterberg et al. 2016; Dhiman et al. 2020) as an expression of axonal damage (Gaetani et al. 2019). Our objectives were dual. Investigate the topographic relationship between these indicators and the metabolism of glucose in the brain, on the one hand. To determine if and to what extent these correlated locations relate to still largely unaltered regions or overlap with hypometabolic zones as determined from comparing MCI-AD patients with a group of healthy subjects. Overall, the alterations of different brain areas related to these proteins suggest they express distinct pathophysiological processes occurring in prodromal AD. Briefly, we observed negative correlations between the Ng and -syn concentrations in the CSF and the metabolism of the left precuneus/posterior cingulate

cortex (PC/PCC), which largely comprised regions of low metabolism. The metabolic correlates of β-syn and NfL regarded either the left or right lateral temporal areas, respectively, with some overlap with hypometabolism only for the β-syn-related volume. This leads us to hypothesize that the levels of i) CSF Ng and α-syn express a hippocampal damage that has already developed and is causing PC/PCC deafferentation and hypometabolism; ii) CSF β-syn may represent the development of synaptopathy in the temporal lobe; and iii) CSF NfL reflect the axonal injury in right temporal regions where neuronal loss is not yet manifest.

3. Reciprocal incremental value of [^{18}F]-FDG-PET and CSF biomarkers in MCI patients suspected for Alzheimer's disease and inconclusive first biomarker

Massa F[1], Farotti L[2], Eusebi P[3,4], Capello E[5], Dottorini ME[6], Tranfaglia C[6], Bauckneht M[7], Morbelli S[7,8], Nobili F[1,5], Parnetti L[2]

[1] Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health (DINOGMI), University of Genoa, Genoa, Italy
[2] Center for Memory Disorders and Laboratory of Clinical Neurochemistry, Neurology Clinic, University of Perugia, Perugia, Italy
[3] Section of Neurology, Department of Medicine, University of Perugia, Perugia, Italy;
[4] Health Planning Service, Department of Epidemiology, Regional Health Authority of Umbria, Perugia, Italy
[5] Neurology Clinic, IRCCS Ospedale Policlinico San Martino, Genoa, Italy
[6] Nuclear Medicine Unit, "S. Maria della Misericordia" Hospital, Perugia, Italy
[7] Nuclear Medicine Unit, IRCCS Ospedale Policlinico San Martino, Genoa, Italy
[8] Department of Health Sciences (DISSAL), University of Genoa, Italy

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Abstract

Background. In Alzheimer's disease (AD) diagnosis, both cerebrospinal fluid (CSF) biomarkers and FDG-PET give sometimes inconclusive results. To evaluate the incremental diagnostic value of FDG-PET over CSF biomarkers, and *vice versa*, in patients with Mild Cognitive Impairment (MCI) and suspected AD, in which the first biomarker resulted inconclusive.

Methods: A consecutive series of MCI patients was retrospectively selected from two Memory Clinics where, as per clinical routine, either the first biomarker choice is FDG-PET and CSF biomarkers are only used in patients with uninformative FDG-PET, or viceversa. We defined criteria of uncertainty in interpretation of FDG-PET and CSF biomarkers, according to current evidence. The final diagnosis was established according to clinical-neuropsychological follow-up of at least one year (mean 4.4±2.2).

Results. When CSF was used as second biomarker after FDG-PET, 14 out of 36 (39%) received informative results. Among these 14 patients, 11 (79%) were correctly classified with respect to final diagnosis, thus with a relative incremental value of CSF over FDG-PET of 30.6%. When FDG-PET was used as second biomarker, 26 out of 39 (67%) received informative results. Among these 26 patients, 15 (58%) were correctly classified by FDG-PET with respect to final diagnosis, thus with a relative incremental value over CSF of 38.5%.

Conclusions. Our real-world data confirm the added values of FDG-PET (or CSF) in a diagnostic pathway where CSF (or FDG-PET) was used as first biomarkers in suspected AD. These findings should be replicated in larger studies with prospective enrolment according to a Phase III design.

1. Introduction

Mild Cognitive Impairment (MCI) is a challenging condition due to its heterogeneous clinical presentation and pathophysiology (Stephan et al. 2012; Petersen et al. 2014). The risk of progression to dementia mainly depends on the underlying etiology (Albert et al. 2011a) and current studies are aimed at defining those biomarkers able to elucidate the specific pathophysiology, guiding clinicians to the best management in terms of diagnosis, prognosis, care, and early therapies (Berk et al. 2014). Such biomarkers are spread among centers dedicated to neurodegenerative diseases and several data are available about their specific features and accuracy (see for a review Fiandaca et al. 2014) [5], although their clinical validation is still incomplete (Frisoni et al. 2017).

Moreover, evidence about the best cost-effective and accurate flowchart guiding the use of biomarkers is still incomplete. The studies comparing biomarker performances do not definitely demonstrate the superiority of one over the others (Herukka et al. 2017; Simonsen et al. 2017). As an example, a trend in favor of a higher accuracy of FDG-PET than CSF biomarkers reported by some papers (Shaffer et al. 2013; Prestia et al. 2015; Caminiti et al. 2018) has not been confirmed by a meta-analysis with pathological confirmation, reporting instead a similar accuracy (Cure et al. 2014).

Although MCI can evolve to different types of dementia, most studies focused on Alzheimer's disease (AD) (Albert et al. 2011a) and how biomarkers of amyloidosis, tauopathy, and neurodegeneration are useful to track the disease at the MCI stage (Jack et al. 2016a). Several studies have shown that the combinatorial use of these biomarkers adds diagnostic value to the use of a single one (Bouwman et al. 2007; Vemuri et al. 2009; Choo et al. 2013; Prestia et al. 2013). However, performing all (or most) biomarkers in a MCI patient is not cost-effective since the majority of patients can be diagnosed accurately with the use of a single biomarker and only those with inconclusive results may have access to a second assessment.

In this scenario a stepwise approach could reach a cost-effective and specific diagnosis. There is active debate on which biomarker should come first and precisely for which clinical presentation. CSF biomarkers are increasingly proposed, wherever

available, if the main clinical suspicion is AD whereas FDG-PET is mainly suggested if other diagnoses are at least as likely as AD (Boccardi et al. 2019; Chételat et al. 2019). Both CSF biomarkers and FDG-PET have high diagnostic accuracy and are indeed recommended by scientific societies (Herukka et al. 2017; Arbizu et al. 2018; Nobili et al. 2018a) as a first-line biomarker in MCI patients while amyloid PET and dopaminergic imaging would have a more limited use in specific conditions (Johnson et al. 2013; Boccardi et al. 2019).

Waiting for shared and evidence-based recommendations on their alternative use as a first-line biomarker, in the real-life the first choice often mainly relies on the confidence of the clinician, local facilities, waiting lists, expertise and performance of local laboratories. In the case the first choice gave inconclusive results thus the other may be used to achieve a correct diagnosis but their reciprocal relative incremental diagnostic value when the first one is inconclusive is poorly known and partly conflicting.

Indeed, the higher predictive value of FDG-PET toward conversion to AD dementia in MCI patients reported in the ADNI population (Shaffer et al. 2013) has not been confirmed by Choo et al. (Choo et al. 2013) who reported a similar power for both FDG-PET and total Tau (t-Tau) whereas in another study using a stepwise approach the hyperphosphorilated Tau (p-Tau) achieved the best result (Lange et al. 2017).

Rather than evaluating accuracy of individual biomarkers that has already been the object of previous investigations (Fiandaca et al. 2014) in this study we evaluated the relative and reciprocal incremental diagnostic value in the attempt to establish the etiological diagnosis of CSF biomarkers over FDG-PET, and *viceversa*, only in those patients with inconclusive results after examination of the first biomarker. To this purpose we retrospectively selected data acquired for clinical purpose in naturalistic populations in two memory clinics used to apply as per clinical routine either the CSF biomarkers as a first approach and then FDG-PET when the former gives inconclusive results, or *viceversa*. In these two cohorts we first reported when the second biomarker came to a likely diagnosis and then computed how many of such diagnoses were confirmed at clinical follow-up, used as the gold standard.

2. Material and methods

2.1. Patients

Patients were retrospectively and consecutively enrolled from two memory clinics. As per clinical routine, in a patient with MCI and suspected AD the first choice to try to establish an etiological diagnosis is FDG-PET in Genoa and CSF biomarkers are only used in patients with an uninformative FDG-PET; the opposite happens in Perugia. The retrospective patient selection criteria were that i) the first biomarker was assessed soon after (i.e., within 2 months) the initial clinical evaluation because of an uncertain diagnosis following standard clinical-neuropsychological assessment and MRI; ii) AD was one of the possible diagnosis; iii) the second biomarker was performed because the first one gave uncertain results (*uninformative)* and iv) patients were clinically followed-up (FU) for at least one year. It should be underscored that we did not select those patients whose differential diagnosis did not include AD.

All patients fitted the diagnostic criteria for MCI (Petersen et al. 2009), including amnesic and non-amnesic MCI, either single- or multi-domain. Activities of daily living (ADL) and instrumental ADL (IADL) were assessed through clinical interview and formal questionnaires, and clinical dementia rating (CDR) scale had to be 0.5. Patients underwent standard clinical and neuropsychological assessment exploring verbal and spatial memory, executive functions, attention, language, and visuo-construction, according to local clinical routine. The neuropsychological batteries included standard tests with available normative values in local language and only slightly differed between centers. Brain MRI (or CT when MRI was unfeasible) was available in all patients and allowed to exclude other causes of cognitive impairment, including vascular cognitive impairment (Gorelick et al. 2011). All other causes of secondary cognitive impairment were excluded based on clinical examination and blood tests.

From January 2012 to May 2018, we identified seventy-five patients (36 males, 39 females; mean age 70.2±7.3; mean MMSE score 26.6±2.5), including thirty-six patients enrolled in Genoa and thirty-nine in Perugia. We named the two cohorts according to the second biomarker used (i.e., CSF2 when FDG-PET came first and

was uninformative, and FDGPET2 when CSF biomarkers came first and were uninformative).

Table 1. Main clinical and demographic characteristics and CSF biomarkers values in CSF2 and FDGPET2 groups

	CSF2 group (n = 36)	FDGPET2 group (n = 39)	p
Age (yrs)	70.9 ± 7.6	69.5 ± 7.0	n.s.
Sex (M/F)	16/20	20/19	n.s.
MMSE score	27.2 ± 2.4	26.1 ± 2.6	n.s.
CSF $A\beta_{42}$ (pg/mL)	682.2 ± 284.8	682.0 ± 333.6	n.s.
CSF t-Tau (pg/mL)	515.9 ± 368.9	413.9 ± 216.5	n.s.
CSF p-Tau (pg/mL)	66.1 ± 31.5	58.9 ± 20	n.s.
Δ-Time (yrs)	0.9 ± 0.5	0.3 ± 0.2	0.01
aMCI / naMCI	33 / 3	24 / 15	0.001
sdMCI / mdMCI	12 / 24	10 / 29	n.s.

Abbreviations: CSF, cerebrospinal fluid; CSF2, CSF as second biomarker; FDGPET2, [18F]-Fluorodeoxyglucose Positron Emission Tomography (FDG-PET) as second biomarker; MMSE, Mini Mental Score Examination; Aß, amyloid-beta; t-Tau, total tau; p-Tau, hyperphosphorylated Tau; Δ-Time, mean time gap between the two biomarkers; aMCI, amnestic Mild Cognitive Impairment; naMCI, non-amnestic MCI; sdMCI, single domain MCI; mdMCI, multi-domain MCI

The final diagnosis was established according to the last available FU visit after at least one year (mean 4.4±2.2 years, range 1.02-10.61), according to current diagnostic criteria in those patients converting to dementia (Litvan et al. 1996; Gorno-Tempini et al. 2011; McKhann et al. 2011; Rascovsky et al. 2011; Postuma et al. 2015; Jack et al. 2016b; Respondek et al. 2017; McKeith et al. 2017). The managing clinician was a neurologist with expertise in dementia and was aware of biomarker results. Conversion to dementia was ascertained by clinical interview with patients and caregivers and by means of the ADL, IADL, and CDR scales.

2.2. CSF biomarkers

According to the current standard operative procedures (Teunissen et al. 2009) CSF samples (6-8 mL) were collected by lumbar puncture in the L3-L4 or L4-L5 interspace and the procedure was always performed early in the morning. CSF was collected in sterile polypropylene tubes, centrifuged for 10 min at 4000g at 4 °C, and the aliquots stored in polypropylene tubes at −80 °C until analysis, to ensure long-term stability of proteins. CSF biomarkers included $A\beta_{42}$, t-Tau (total-Tau), and p-Tau (phosphorylated at threonine 181 or p-Tau181p) expressed in pg/mL, that were measured in both centers using commercially available enzyme-linked immunosorbent assays (ELISAs) kits (INNOTEST Fujirebio Europe, Gent, Belgium). Cut off-values have been computed according to ROC analysis and by using the highest Youden index in a large population of patients with longitudinal evaluations, diagnosed with AD and non-AD dementias, and neurological controls.

2.3. FDG-PET

PET scan acquisitions were performed in both centers following standardized procedures, according to the European Association of Nuclear Medicine (EANM) guidelines (Varrone et al. 2009).-After at least 6-hour fasting, blood glucose level was measured and was lower than 7.8 mmol/l. 185 – 250MBq of [^{18}F]-FDG were injected via a venous cannula after a 10-minute rest in a silent and obscured room, with closed eyes and unplugged ears. Subjects stayed in the room for 30 min, then they moved to the PET room where scanning started approximately 45 min. after the injection, lasting 15 min. Images were acquired by a Siemens Biograph 16 (Genoa) and a Discovery GE Healthcare ST (Perugia) PET/CT systems. Images were reconstructed with an ordered subset-expectation maximization algorithm following the standard protocols used for clinical purposes and embedded in the equipment workstations. Attenuation correction was based on CT scan.

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

3. Data analysis and interpretation

3.1. CSF biomarkers

Due to small, negligible variation of the normal reference thresholds (within a 5% range) between the two Centers, we decided to choose the Perugia cutoffs. However, we wanted to further check whether the results of analyses would have substantially changed by applying the Genoa thresholds to CSF2 group (entirely made up by Genoa patients) and we obtained that a negligible percentage of patients changes category, which does not affect the results. Therefore, we choose to use the Perugia thresholds taking into consideration that this expert center regularly participates in the Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers.

Cut-offs were as follows: $A\beta_{42}$ 550 pg/mL, t-Tau 400 pg/mL, p-Tau 65 pg/mL. Details of CSF biomarker results as well as borderline or uninformative results are reported in Table 2 (examples in Fig.1). In this last instance of uninformative results we computed the $A\beta_{42}$/pTau ratio (cut-off 4.52, according to local normative data) to verify whether some more patient could be identified as affected by AD pathology, beyond the results of individual biomarkers (Welge et al. 2009).

Table 2. Criteria for interpretation of CSF biomarkers

CSF markers cutoffs	Uninformative CSF profile
$A\beta_{42}$> 550 pg/mL: normal (-) $A\beta_{42}$ 495-550 pg/mL: uncertain (+*) $A\beta_{42}$ < 495 pg/mL: abnormal (+) **t-Tau < 400 pg/mL: normal (-)** t-Tau 400-440 pg/mL: uncertain (+*) t-Tau > 440 pg/mL: abnormal (+) **p-Tau < 65 pg/mL: normal (-)** p-Tau 65-72 pg/mL: uncertain (+*) p-Tau > 72 pg/mL: abnormal (+)	$A\beta_{42}$ (+ or +*)/ p-Tau (-) / t-Tau (-) $A\beta_{42}$ (+ or +*) / p-Tau (+ or +*) / t-Tau (-) $A\beta_{42}$ (+ or +*) / p-Tau (-) / t-Tau (+ or +*) $A\beta_{42}$ (-) / p-Tau (+ or +*) / t-Tau (-) $A\beta_{42}$ (-) / p-Tau (-) / t-Tau (+ or +*) $A\beta_{42}$ (-) / p-Tau (+ or +*) / t-Tau (+ or +*) $A\beta_{42}$ (+*) / p-Tau (+*) / t-Tau (+*)

*A “grey zone” including +10% of t-Tau and p-Tau values and -10% of $A\beta_{42}$ value was considered as uncertain (Molinuevo et al. 2014). Abbreviations: CSF, cerebrospinal fluid; Aß, amyloid beta; t-Tau, total tau; p-Tau, hyperphosphorylated Tau

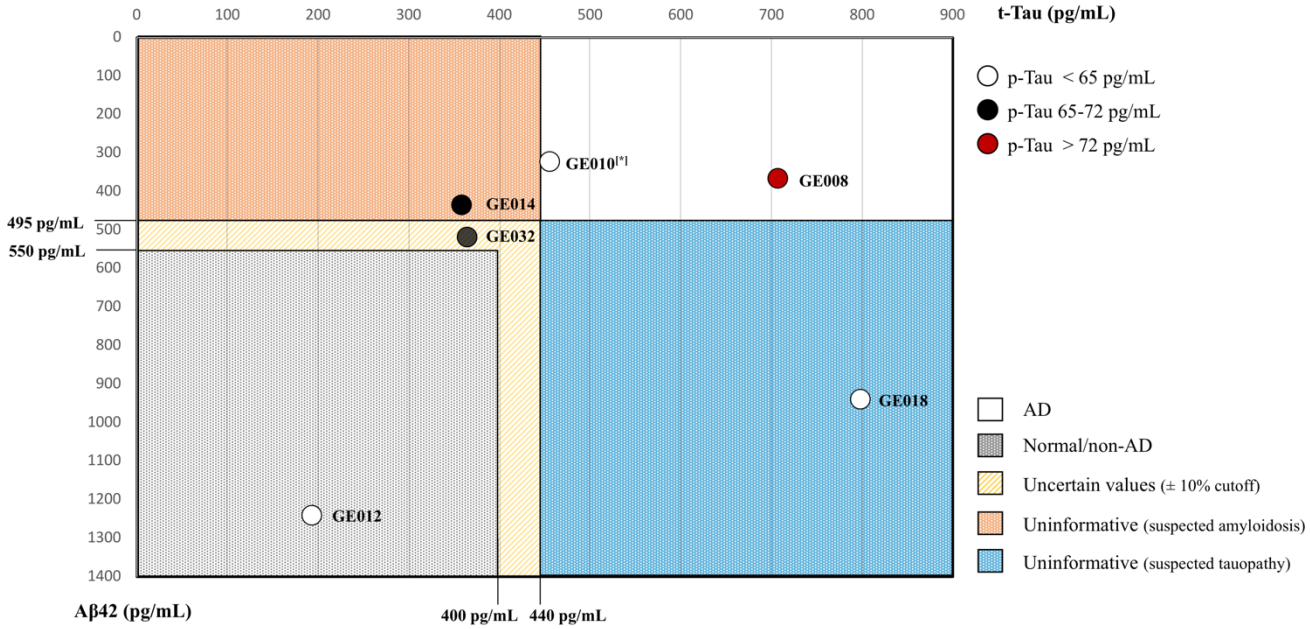

Figure 1. Examples of CSF biomarkers profiles in some patients of CSF2 group are shown with areas of different colours, according to the cut-offs. Each patient is expressed as a circle with different colors (white, black and red) which are determined by the value of p-Tau with respect to the cut-off.

Areas of interpretation of biomarkers (coloured squares) are: white (AD profile), grey (normal), yellow (uncertain area due to borderline values with respect to the cut-offs: +10% for p-Tau and t-Tau and -10% for $A\beta_{42}$), orange (uninformative, with abnormal $A\beta_{42}$, normal t-Tau and p-Tau) and light blue (uninformative, with abnormal t-Tau, normal $A\beta_{42}$ and p-Tau).

[*] Notably, patient GE010 shows both abnormal $A\beta_{42}$ and t-Tau, though the normality of p-Tau leads to a conflicting result which is not classifiable as AD (uninformative analysis).

Abbreviations: CSF2, CSF as second biomarker; GE, Genoa cohort; CSF, cerebrospinal fluid; Aß, amyloid-beta; t-Tau, total tau; p-Tau, hyperphosphorylated Tau; AD, Alzheimer's disease.

3.2. [^{18}F]-FDG-PET

Scans were first visually and independently evaluated by two nuclear medicine experts (S.M. in Genoa, C.T. in Perugia), blinded to clinical history and CSF biomarker results. In fact, reading by experts is recommended by the EANM procedural guidelines (Varrone et al. 2009) and has been shown to be highly accurate (Morbelli et al. 2015a). Their judgment had to cover three options, according to brain metabolism distribution: i) normal (i.e., not compatible with either AD or other neurodegenerative diseases); ii) scan compatible with AD, which included either typical AD (clear hypometabolism in at least one brain region among precuneus/posterior cingulate and posterior temporo-parietal cortex) (Morbelli et al. 2015b) or atypical AD (left temporo-parietal cortex for logopenic variant AD (Gorno-Tempini et al. 2011); parieto-occipital cortex for posterior cortical atrophy (PCA) (Nestor et al. 2003); posterior temporo-parietal cortex plus frontal lobe involvement for frontal variant AD (Woodward et al. 2015)); iii) not compatible with AD but abnormal, when the pattern of hypometabolism involved other cortical areas and was inconsistent with those in ii). In this last instance the experts were asked to suggest an alternative diagnosis based on the metabolic pattern, also following the procedure adopted in previous papers (Grimmer et al. 2016) – i.e., behavioral variant frontotemporal dementia (bvFTD) (Rascovsky et al. 2011; Arbizu et al. 2018), semantic and non-fluent variants of frontotemporal lobe degeneration (Gorno-Tempini et al. 2011; Bouwman et al. 2018), dementia with Lewy bodies (DLB) (McKeith et al. 2017), corticobasal syndrome (CBS) and progressive supranuclear palsy (PSP) (Walker et al. 2018).

When the evaluation was discordant between the two experts, we computed the PALZ score (PMOD® Alzheimer's Discrimination Tool) obtained from semi-quantitative comparison of individual FDG-PET data with those from the normal reference population embedded in the software (cutoff > 11,090) (Herholz et al. 2002). If the PALZ score was above the cutoff (>11090) and the two experts agreed that the resulting statistical maps identified an AD hypometabolic pattern then the scan was judged to be positive for AD (example in Fig. 2-A). Otherwise FDG-PET was considered as uninformative if i) the PALZ score was below the cutoff (< 11090) (example in Fig. 2-B) or ii) the PALZ score was >11090 but the two experts did not

identify an AD typical hypometabolic pattern by evaluating the topography and extent of significant hypometabolic regions as showed by the statistical map (example in Fig. 2-C). To summarize, FDG-PET was 'uninformative' when it was abnormal but neither an AD-typical hypometabolic pattern was evidenced by the experts and supported by PALZ score, nor other specific hypometabolic pattern for a non-AD neurodegenerative disease was found.

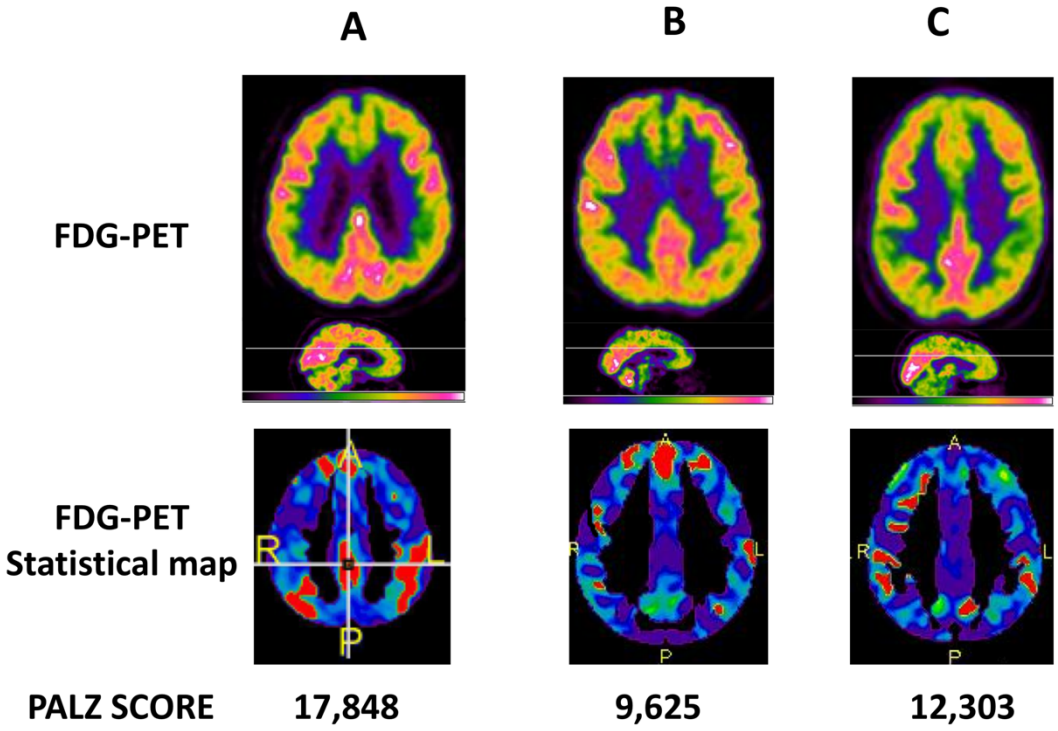

Figure 2. Examples of reconstructed FDG-PET images showed in a trans-axial slice (according to the color bar below: white, red and yellow as 'hot' colors, green, blue and black as 'cold' colors) (top) with the corresponding trans-axial slice as shown by voxel-based analysis compared with healthy controls, corrected for age (bottom) where the significant hypometabolic voxels are reported in red.
In these three examples the two expert readers disagreed about the presence of an AD hypometabolic pattern and thus voxel-based statistical comparison was performed by means of the PALZ score computation embedded in the PMOD® software (see text for further details). A) in this patient the PALZ score was positive for AD and the two experts recognized an AD hypometabolic pattern in the statistical maps; B) in this case the PALZ score was negative for AD; C) in this last case the PALZ score was positive for AD but the two experts did not recognize an AD-typical hypometabolic pattern.

3.3. Diagnostic pathways

The possible pathways differed between CSF2 and FDGPET2 cohorts because pathway i) in FDGPET2 cohort ruled out all neurodegenerative conditions and not only AD as in cohort CSF2, and pathway iii) was limited to 'uninformative' in CSF2 cohort without the possibility to suggest an alternative diagnosis, while in FDGPET2 an alternative diagnosis could be suggested. The diagnoses suggested after obtaining the results of the second biomarker were compared with the final clinical diagnosis at FU taking into account all biomarker results that was regarded as the 'gold standard'.

3.4. Statistics

Demographical features, MMSE score, clinical presentation (amnestic or non-amnestic MCI, single or multidomain) and CSF profile were compared between the two cohorts (CSF2 and FDGPET2) (t-test or chi-square test).

We then focused on the performance of the two biomarkers with a three-step computation. In either CSF2 or FDGPET2 cohort, respectively, we first computed the number (percentage) of patients in whom the second biomarker gave an informative result with respect to the CSF2 or FDGPET2 cohort. Second, we computed the number (percentage) of patients with an informative second biomarker whose diagnosis was confirmed at FU. Third, we expressed the number (percentage) of these last patients with respect to the CSF2 or FDGPET2 cohort, allowing us to compute the relative incremental value for each biomarker over the other one, as follows: relative incremental value (%) = (diagnosis suggested by the second biomarker and clinically confirmed at FU / all patients with *uninformative* first biomarker)*100.

4. Results

The two cohorts showed similar age, sex distribution, MMSE score and CSF biomarkers values and only differed for the time lapse between the two biomarkers performance and for the distribution of amnestic and non-amnestic MCI (Table 1). During follow up 45/75 patients (60%) converted to and were diagnosed with a specific form of dementia, considering 18 in CSF2 group (16 AD, 2 bvFTD) and 27 in FDG2 group (17 AD, 9 bvFTD, 1 PSP).

4.1. CSF2 cohort (Table 3a)

CSF biomarkers gave an informative result in 14/36 patients (38.9%). In detail, CSF profile was normal (i.e., not compatible with AD, non-AD) in ten patients, compatible with AD in four patients, and was uninformative in the remaining twenty-two in whom also the $A\beta_{42}$/pTau ratio was not confirmatory of AD. During follow up evaluations among these 14 patients, 11 (78.6%) were confirmed at FU; indeed, all the four patients with a CSF profile compatible with AD were confirmed as affected with AD at FU, while three among the ten patients with a normal CSF were diagnosed as AD and seven were confirmed as non-AD (including three patients with masked, sub-threshold depression, two FTD phenocopies and two patients with obstructive sleep apnea syndrome).

Among the 22/36 patients with an uninformative CSF profile, nine patients had a final diagnosis of AD at FU and seven of non-AD pathologies (two with bvFTD, two with masked depression, three with amnestic MCI (aMCI) satisfying the criteria for suspected non-Alzheimer pathology (SNAP) according to CSF profile (Jack et al. 2016b), whereas six patients remained undiagnosed and phenotypically described according to their cognitive profile (Petersen et al. 2009), i.e., three with single-domain aMCI, two with multi-domain aMCI and one with non-amnestic MCI (naMCI).

Thus, 11/36 cases were clarified by CSF biomarkers and then clinically confirmed at FU (four with AD and seven non-AD final diagnoses) leading to a relative incremental diagnostic value of 30.6% of CSF biomarkers with respect to an uninformative FDG-PET to define an MCI as due to AD or non-AD.

Table 3a. Summary of CSF2 group according to interpretation after second biomarker and the definite diagnoses at follow-up.

CSF2					
Pt	**AGE (y)**	**MMSE**	**MCI phenotype**	**CSF results**	**FINAL DIAGNOSIS**
GE002	64.4	30	aMCIsd	NORMAL	AD
GE007	71.8	25	aMCIsd	NORMAL	AD
GE012	62.7	30	aMCImd	NORMAL	bvFTD phenocopy
GE016	65.6	29	aMCImd	NORMAL	DEP
GE020	73.8	26	naMCImd	NORMAL	OSAS
GE023	80.3	26	naMCImd	NORMAL	DEP
GE025	74.7	25	aMCImd	NORMAL	DEP
GE028	66.0	27	aMCIsd	NORMAL	AD
GE033	82.5	23	aMCIsd	NORMAL	bvFTD phenocopy
GE034	68.4	25	aMCImd	NORMAL	OSAS
GE008	73.1	26	aMCImd	AD	AD
GE024	72.7	29	aMCImd	AD	AD
GE030	66.9	20	aMCImd	AD	fvAD
GE035	77.7	25	aMCImd	AD	fvAD
GE001	65.3	29	aMCIsd	uninformative	aMCIsd
GE003	72.4	28	aMCImd	uninformative	aMCImd - SNAP
GE004	67.3	29	aMCIsd	uninformative	AD
GE005	73.0	29	aMCIsd	uninformative	DEP
GE006	72.6	28	aMCImd	uninformative	bvFTD
GE009	69.3	28	aMCImd	uninformative	AD
GE010	68.9	27	aMCImd	uninformative	fvAD
GE011	67.7	25	aMCImd	uninformative	AD
GE013	71.7	29	aMCImd	uninformative	aMCImd - SNAP
GE014	66.3	30	naMCImd	uninformative	naMCImd
GE015	82.1	29	aMCImd	uninformative	aMCImd
GE017	72.8	30	aMCIsd	uninformative	aMCIsd
GE018	66.4	25	aMCIsd	uninformative	aMCIsd
GE019	47.6	27	aMCImd	uninformative	DEP
GE021	76.3	29	aMCImd	uninformative	AD
GE022	76.1	27	aMCImd	uninformative	aMCImd - SNAP
GE026	81.1	29	aMCImd	uninformative	AD
GE027	78.0	22	aMCImd	uninformative	bvFTD
GE029	74.2	27	aMCIsd	uninformative	AD
GE031	71.7	29	aMCIsd	uninformative	AD
GE032	81.7	30	aMCIsd	uninformative	AD
GE036	50.4	26	aMCImd	uninformative	aMCImd

CSF2, CSF as second biomarker; Pt, patient; GE, Genoa cohort; MMSE, Mini Mental Score Examination score; CSF diagnosis, diagnosis suggested by CSF biomarker; FINAL DIAGNOSIS, diagnosis at follow-up; AD, Alzheimer's disease; fvAD, frontal variant AD; aMCIsd, amnestic Mild cognitive impairment, single-domain; aMCImd, amnestic Mild cognitive impairment, multi-domain; naMCImd, non-amnestic Mild cognitive impairment, multi-domain; bvFTD, behavioral variant-frontotemporal dementia; DEP, masked or sub-threshold depression; OSAS, obstructive sleep apnea syndrome; SNAP, suspected non-Alzheimer pathology.

4.2. FDG-PET2 cohort (Table 3b)

FDG-PET gave informative results in 26/39 patients (66.7%). In detail, FDG-PET was normal in seven patients, showed a typical hypometabolic pattern for AD in twelve patients, and was not compatible with AD but showed a hypometabolic pattern suggesting another disease (i.e., FTD) in seven patients.

During follow up evaluations among these 26 patients, 15 (57.7%) were confirmed at FU – i.e. 10/12 patients with an AD typical hypometabolic pattern and 5/7 with a hypometabolic pattern suggesting FTD. On the other hand, two/12 patients with an AD hypometabolic pattern were diagnosed as bvFTD while in two patients the diagnosis suggested by FDG-PET (i.e., bvFTD and svPPA) was not confirmed at FU. Moreover, among the 7 patients with a normal scan, at FU three were diagnosed with AD, one with bvFTD and three remained undiagnosed.

Among the 13 patients with an uninformative FDG-PET, at FU three patients had a final diagnosis of AD, two patients received other diagnoses (one PSP, one bvFTD), two patients were labeled as SNAP while six remained patients were only labeled according to the MCI subtype.

Thus, in 15/39 patients a correct diagnosis was achieved by FDG-PET with a relative incremental value of 38.5% with respect to an uninformative CSF analysis to define MCI due to a specific neurodegenerative disease.

We lastly compared the main demographic and clinical characteristics between the 26/75 patients (34.7%) in whom the second biomarker allowed a corrected diagnosis as confirmed by the clinical FU (11 in CSF2 and 15 in FDG2), with the remaining 49 patients. We found that the MMSE score was significantly ($p<0.05$) lower in these 26 patients (mean MMSE score 25.8±2.6) as compared to the remaining ones (mean MMSE score 27.0±2.4). No other significant difference was found as for age, sex, time gap between biomarkers, follow up time, MCI phenotype or CSF biomarker values (data not shown).

Table 3b. Summary of FDGPET2 group according to diagnosis suggested by FDG-PET as second biomarker and the definite diagnoses at follow-up.

FDGPET2					
Pt	**AGE (y)**	**MMSE**	**MCI phenotype**	**FDG-PET diagnosis**	**FINAL DIAGNOSIS**
PG001	62.5	28	aMCIsd	NORMAL	aMCIsd
PG007	75.1	21	aMCImd	NORMAL	bvFTD
PG013	72.8	28	naMCIsd	NORMAL	AD
PG028	67.3	29	naMCImd	NORMAL	AD
PG029	72.7	28	aMCImd	NORMAL	AD
PG031	64.7	26	aMCImd	NORMAL	aMCImd
PG036	52.6	30	aMCImd	NORMAL	aMCImd
PG002	65.7	26	aMCIsd	AD	bvFTD
PG005	63.7	22	aMCImd	AD	AD
PG006	61.0	23	aMCImd	AD	AD
PG010	69.6	28	aMCIsd	AD	AD
PG012	66.2	23	aMCImd	AD	bvFTD
PG025	71.5	27	aMCIsd	AD	AD
PG027	57.8	29	aMCImd	AD	AD
PG032	75.7	21	aMCImd	AD	AD
PG033	68.5	27	naMCImd	AD	AD
PG034	82.4	28	naMCImd	AD	AD
PG038	75.0	27	naMCImd	AD	AD
PG039	56.9	26	aMCImd	AD	AD
PG008	73.7	26	naMCIsd	NON-AD (bvFTD)	bvFTD
PG009	65.1	29	naMCIsd	NON-AD (bvFTD)	bvFTD
PG011	81.4	24	naMCImd	NON-AD (bvFTD)	bvFTD
PG017	72.7	25	aMCImd	NON-AD (bvFTD)	bvFTD
PG021	77.8	24	aMCImd	NON-AD (bvFTD)	bvFTD
PG023	72.2	26	aMCImd	NON-AD (svPPA)	AD
PG024	77.0	25	naMCImd	NON-AD (bvFTD)	PD-MCI
PG003	57.7	23	naMCImd	uninformative	naMCImd
PG004	71.8	29	naMCImd	uninformative	naMCImd
PG014	74.9	28	aMCImd	uninformative	aMCImd - SNAP
PG018	75.7	27	aMCIsd	uninformative	AD
PG019	72.4	28	aMCImd	uninformative	aMCImd
PG022	65.4	29	naMCIsd	uninformative	naMCIsd
PG026	57.6	26	aMCImd	uninformative	aMCImd - SNAP
PG030	74.1	20	aMCImd	uninformative	aMCImd

FDGPET2, [^{18}F]-Fluoro-deoxyglucose Positron Emission Tomography (FDG-PET) as second biomarker; PG, Perugia cohort; FDG-PET diagnosis, diagnosis suggested by FDG-PET; svPPA, semantic variant-Primary Progressive Aphasia; PD, Parkinson's disease; PSP, progressive supranuclear palsy.
Other abbreviations as in Table 3a.

5. Discussion

We assessed the relative added diagnostic value of performing a second biomarker when the previous work-up, including clinical-neuropsychological evaluation, morphologic neuroimaging and a first-choice biomarker (CSF for Perugia, FDG-PET for Genoa) was inconclusive to define a MCI of a suspected AD origin.

Basing on the confirmed diagnoses at a mid-term follow-up (mean 4.4 years) used as the gold-standard, the relative incremental diagnostic values of CSF biomarkers and FDG-PET over one another was 30.6% and 38.5%, respectively (average 34.7% in the whole cohort), with a small advantage for FDG-PET.

Available literature data are somehow conflicting on the superiority of one biomarker over the other. In one study, both FDG-PET and total Tau (t-Tau) reached a similarly high predictive power toward conversion of MCI patients to AD dementia (Choo et al. 2013). In another study, hyperphosphorylated tau (p-tau) was highly sensitive to subsequent cognitive decline while FDG-PET was superior in predicting conversion to dementia in MCI patients (Fellgiebel et al. 2007). In the ADNI population, accuracy in predicting conversion to AD dementia was higher for FDG-PET (87.4%) than for CSF biomarkers (74.1%) and MRI (69.5%) with a combinatorial accuracy of 92.1% (Shaffer et al. 2013).

However, in those studies FDG-PET and CSF biomarkers, as well as other biomarkers, were performed and evaluated altogether rather than in a progressive fashion only when the first biomarker gave inconclusive results. Instead, our work focused on a 'sequential' approach in the decision-tree of MCI suspected for AD diagnosis, rather than a 'in parallel' scheme, in a similar way as in the study finding a 35% relative incremental diagnostic value of amyloid PET after an inconclusive CSF (Weston et al. 2016), practically an identical rate of increase as in the present study. Also, Lange et al. (Lange et al. 2017) meaningfully followed a stepwise order (namely ADAS-13 score, hippocampal volume (HV), CSF and FDG-PET) and showed that CSF p-Tau provided the best incremental risk stratification when added to ADAS-13 score with respect to HV and FDG-PET, while FDG-PET used in the second step outperformed HV in MCI subjects with relatively preserved cognition (ADAS-13

score <18). Moreover, CSF $A\beta_{42}$ provided additional risk stratification in MCI subjects with increased CSF p-Tau.

Concerning the magnitude of relative incremental diagnostic value, whether the percentage of about one third of patients clarified by the second-line biomarker should be considered clinically relevant thus justifying the costs and the (mini)invasiveness of the procedures or not, this is matter of active investigation and is beyond the aims of the present study. Anyway, our results are in line with a study showing that adding CSF biomarkers to FDG-PET led to a small but measurable increase in diagnostic accuracy (Zhang et al. 2011). Also, an increase in diagnostic accuracy was shown using not precisely the same biomarkers as ours. A benefit of combining MRI and FDG-PET in predicting conversion to AD dementia with respect to the single biomarker was reported (Walhovd et al. 2010) and a further 6% AUC value increase was demonstrated from the best single (t-Tau, AUC 0.77) to the best three-predictors combination model (t-Tau/HCV/CDR-sum of boxes, AUC = 0.83) in predicting conversion to AD dementia (Frölich et al. 2017).

Taken together, these as well as our data suggest that using biomarkers with a progressive approach allows to increase the diagnostic accuracy although a non-trivial part of patients remains undiagnosed. This means that those patients leading to clinician uncertainty after basal clinical-neuropsychological assessment, MRI and a first biomarker are likely to be the most complex ones, including those with mixed pathologies, confounding factors, or atypical presentations, or a mix of these conditions (Jellinger and Attems 2010; Carotenuto et al. 2012; Dubois et al. 2014a). To reinforce this interpretation, in 20 patients of the whole our cohort (26.7 %) an etiologic diagnosis was not achieved even at follow-up, as MCI patients did not convert to dementia and, furthermore, no specific suggestive features about the underlying etiology could be unveiled, including 5 of them who satisfied the criteria for SNAP according to CSF profile (Jack et al. 2016b). The only factor that distinguished those patients taking advantage from a second biomarker assessment was a significantly lower MMSE score although the overlap with those remaining undiagnosed was considerable.

Another point of our results deserving discussion is that the majority (65%) of diagnoses suggested by an informative second-line biomarker were confirmed at

follow-up, in this case with an advantage of CSF biomarkers (78.6%) over FDG-PET (57.7%), although the clinician was not blind to the results of biomarker itself because this is a retrospective study with data collected from the real clinical practice. As we lacked pathological confirmation and used the clinical follow-up as the gold-standard, we cannot firmly establish that in the remaining 35% of patients the second biomarker 'was wrong' because we know the clinical diagnosis carries in turn a considerable risk of errors (Beach et al. 2012), especially if we consider that here the final clinical diagnosis tells against the suggestion given by the second biomarker. Moreover, even though mean clinical FU was 4.4±2.2 years, in 11 patients it was less than 2 years (of whom 6 patients < 18 months), so we might have missed some late converters who could have been properly diagnosed with a longer period of observation.

Moving to more technical considerations, as for CSF biomarker a grey zone of 10% above (t-Tau or p-Tau) or below ($A\beta_{42}$) is admitted (Molinuevo et al. 2014; Lewczuk et al. 2015; Simonsen et al. 2017). Accordingly, we considered as uninformative CSF biomarkers when at least one of the three proteins was conflicting with the expected 'AD signature' or included in the grey zone (Molinuevo et al. 2014). Such strategy could be responsible for the high rate of uncertain interpretations (22/36), but was not mitigated by our attempt to overcome this limitation by using the $A\beta_{42}$/pTau ratio. This is in line with the knowledge that almost half of the patients with AD may have conflicting CSF biomarkers results (Alexopoulos et al. 2016), only partly due to a false initial diagnosis or to adopted cutoffs and laboratory performance (Rosén et al. 2015; Herukka et al. 2017).

Indeed, these uncertain cases reflect at least in part the heterogeneous composition of MCI population as low CSF $A\beta_{42}$ may be found in non-AD conditions such as Parkinson's disease and DLB (Kaerst et al. 2013), while Tau levels may be elevated in several other conditions expressing neurodegeneration (t-Tau), tauopathy (p-Tau), or both (van Harten et al. 2011).

Another part of variance may derive from pre-analytical and analytical issues since the sampling was realized in the course of years, and even if collection and analytical procedures were the exactly the same, some practical conditions may have changed, mainly depending on the technical skills which inevitably increase

paralleling experience. Lastly, some variance may derive from suboptimal inter- and intra-center reproducibility using commercial kits (Verwey et al. 2009).

Lastly, we now know that the $A\beta_{42}/A\beta_{40}$ ratio is more accurate than the $A\beta_{42}$ assay alone (Lewczuk et al. 2014; Dorey et al. 2015; Biscetti et al. 2019), but our casuistic was retrospectively collected when only the $A\beta_{42}$ assay was available. It is likely that adding the ratio $A\beta_{42}/A\beta_{40}$ could have solved some more cases, especially those with an uncertain $A\beta_{42}$ value.

Obviously, when using such biomarkers we should have clearly in mind what information we expect from them. As said above, as for clinical routine the two centers have a different use of biomarkers, which mainly depends on a different diagnostic strategy to infer the pathological processes underlying MCI. Notwithstanding, as per recruitment criteria all patients should have had a MCI primarily suspected for AD. Such specific pre-requisite prevented any selection bias and allowed us to specifically focus on the reliability of CSF and [^{18}F]-FDG PET to confirm or rule out AD.

Within the context of current diagnostic criteria (Albert et al. 2011a) our results confirm the high specificity of CSF markers in AD as they are able to simultaneously assess the main pathological features which confer the highest likelihood that AD is the cause of MCI (Dubois et al. 2014b; Jack et al. 2018) Moreover, our results enforced the role of [^{18}F]-FDG PET as a reliable diagnostic tool not restricted only to AD (Nobili et al. 2018a). Thus, as for FDG-PET, the main advantage seems that an alternative diagnosis can be suggested when an AD-like hypometabolic pattern is not found, as in the case of FTD. This further stresses the role of FDG-PET in other etiologies than AD, in keeping with the recent EANM-EAN joint recommendations (Nobili et al. 2018a). On the other hand, FDG-PET is a non-pathology specific marker, thus in some instances the imaging endophenotype may mimic another disease by showing an unusual topographic pattern, and this is likely the explanation of some of the diagnostic mistakes we observed in the present series. Just as an example, the concomitant frontal and parietal hypometabolic pattern can be found in patients with fvAD but also in an occasional patient with FTD in which parietal hypometabolism may be due to the fronto-parietal disconnection through the fronto-parietal pathways (Johnson et al. 1999; Womack et al. 2011; Woodward et al. 2015).

Both for CSF analysis and FDG-PET we are aware of the paramount importance of laboratory and readers expertise in borderline or conflicting results which may predispose to misinterpretation (Herholz 2014; Fourier et al. 2015; Lewczuk et al. 2018). As a consequence, some of the mistakes in the present research may have been derived from analytical issues rather than from the intrinsic limitations of the diagnostic tools *per se*, but we described what happens in clinical practice in two expert centers, even with different field of expertise, thus very close to the real life in most centers worldwide. Also, the different time gap between biomarkers, which was significantly longer when CSF biomarkers were used after FDG-PET, is mainly be due to the reluctant approach (in the past years) of both physicians and patients/caregivers to the lumbar puncture in the Genoa center.

In conclusion, finding the best way to get to the highest diagnostic accuracy by using the least number of biomarkers is of paramount relevance not only for clinicians but also for national healthcare systems, as people with MCI are expected to progressively increase, along with costs given by diagnostic procedures, welfare and hopefully upcoming disease-modifying drugs. The main strength of our work is to have involved naturalistic cohorts, not in the set of clinical trials with selected patients, and thus it is more adherent to the real world; this let us to propose our evidences also in the clinical scenario in which MCI is a diagnostic challenge as a definite diagnosis could not be achieved even after a second-line biomarker evaluation in a substantial part of cases. Anyway, the retrospective nature of our study as well as to have focused on a selected cohort in which the first additional biomarker was unable to clarify the etiology are the main limits which require caution in generalizing the results.

Moreover, Amyloid PET has been routinely used in our centers only for 2-3 years and thus it is unavailable in the vast majority of our cases; consequently, we are far from having a sufficient number of patients to explore its role in this context. Amyloid PET has been reported to better predict AD-dementia conversion than CSF (Ben Bouallègue et al. 2017). Thus, it might be the case that some still uncertain diagnoses even after CSF analysis and [^{18}F]-FDG PET or some cases which were not properly classified could have been clarified by amyloid PET, as well as by the ratio Aβ1-42/Aβ1-40, as discussed before.

The utility of our study is to have assessed the relative incremental value of two of the most used and widespread biomarkers in the diagnostic workup of MCI and to have highlighted the complexity of uncertain diagnoses. In particular we found a small advantage for FDG-PET with respect to CSF in achieving the most accurate diagnosis; thus, from a practical standpoint we might recommend FDG-PET when the diagnostic scenario has not been narrowed by first-line assessments, and still includes different diseases other than AD; on the other hand adding CSF might be helpful when we need a specific confirm of a justified suspicion of AD which FGD-PET at first was not able to solve.

According to this finding, one may want to access the second biomarker only if the cognitive deficit is more marked and, as a suggestion, if it is further confirmed in subsequent visits in the short term, but this hypothesis requires further studies. Moreover, as we found a prevalence of naMCI in the FDG2 group we hypothesize that a patient with naMCI could more likely have an inconclusive CSF compared to patients with aMCI, the typical presentation of AD and with a higher likelihood to exhibit a 'typical' CSF AD profile. Thus, naMCI patients who are still suspected of AD (as in our cohorts) are usually further assessed with a second biomarker (FDG-PET in our case) in the attempt to achieve an etiological diagnosis. On the other hand, aMCI might be underpinned by different conditions other than AD (such as SNAP, cerebrovascular disease, and even subthreshold depression); thus, an aMCI patient with an inconclusive FDG-PET might need CSF analysis as a second biomarker to suggest or rule out AD. Another unmet need is the potential role of either of the two biomarkers as gate keeper after neuropsychology and MRI, especially taking into account the age. Increasing evidence is supporting the use of amyloid biomarkers for subjects with MCI of suspected AD origin who are younger than 65, due to the high rate of cerebral amyloid pathology of unknown significance in cognitively unimpaired older people (Jansen et al. 2015). However, the specific role for FDG-PET as gate keeper for those who are older has never been investigated and goes beyond the aims of the present study.

Efforts should be directed towards the definition of a diagnostic-tree that considers either the main features of each biomarker, alone or in sequential combination, and a cost-effective approach to identify those patients in which adding

a second biomarker could be really useful. We believe the results of this retrospective and relatively small-number study would deserve larger and prospective studies to assess the reliability and clinical utility of sequential biomarker measurement, according to clinical presentation.

4. Exploring the brain metabolic correlates of process-specific CSF biomarkers in patients with MCI due to Alzheimer's disease: preliminary data

[1]Federico Massa, [2]Steffen Halbgebauer, [2,3]Lorenzo Barba, [2,4]Patrick Oeckl, [2]Nerea Gómez de San José, [5]Matteo Bauckneht, [6]Francesco Lanfranchi, [5]Tiziana Vigo, [1,5]Dario Arnaldi, [1,5]Matteo Pardini, [5,6]Silvia Morbelli, [7]Andrea Chincarini, [8]Henryk Barthel, [2,3]Markus Otto and [1,5]Flavio Nobili

[1]Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health (DINOGMI), University of Genoa, Genoa, Italy

[2]Department of Neurology, Ulm University Hospital, Ulm, Germany

[3]Department of Neurology, Martin-Luther-University of Halle-Wittenberg, Halle/Saale, Germany

[4]German Center for Neurodegenerative Diseases (DZNE e.v.), Ulm, Germany

[5] IRCCS Ospedale Policlinico San Martino, Genoa, Italy

[6] Department of Health Science (DISSAL), University of Genoa, Italy

[7] National Institute of Nuclear Physics (INFN), Genoa section, Genoa, Italy

[8] Department of Nuclear Medicine, University Hospital of Leipzig, Leipzig, Germany

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Abstract

Introduction. Cerebrospinal fluid (CSF) biomarkers can reflect several molecular aberrations and pathological changes in Alzheimer's disease (AD), e.g., neuronal death, synaptic and axonal injury. [^{18}]F-fluorodeoxyglucose-PET(FDG-PET) brain metabolism might disclose a peculiar topography when correlated with the levels of some CSF proteins reflecting specific pathological processes.

Methods. We focused on some emergent CSF biomarkers, namely post-synaptic neurogranin (Ng), pre-synaptic α-and β-synuclein (α-and β-syn), and neurofilament light chain (NfL), as a marker of axonal damage, and explored the sites of correlation (volumes of interest, VOIs) of their levels with brain metabolism in a group of 26 patients with prodromal AD (16 females; age 75.4±6.6; MMSE score 26.1±1.9). We further assessed whether-and how extensively- these VOIs overlapped the hypometabolic areas resulting from comparing AD patients with 40 matched healthy controls (HC).

Results. Ng-VOI and α-syn-VOI encompassed left precuneus/posterior cingulate cortex (PC/PCC) and partially overlapped hypometabolism at those sites. β-syn-VOI and NfL-VOI regarded either left or right lateral temporal areas, respectively, with partial overlap with hypometabolism for the β-syn-VOI, whereas the NfL-VOI did not include hypometabolic regions (Figure 1).

Discussion. We speculate that CSF levels of Ng and α-syn express an already established hippocampal damage leading to PC/PCC deafferentation and hypometabolism. β-syn may represent the progression of synaptopathy in the temporal lobe, while NfL the axonal injury in less affected right temporal regions where neuronal loss is still subthreshold. These findings complement the information on the distribution of hypometabolism related to neuronal loss, which differs from the metabolic changes reflecting synaptic or axonal injury.

1. Introduction

In the past years, the research provided a deep insight into the pathogenesis of Alzheimer's disease (AD), and we can now assess many molecular aberrations and pathological changes through cerebrospinal fluid (CSF) analysis. Beyond the core CSF biomarkers of amyloidosis (amyloid β 1-42 normalized for amyloid β 1-40 values, Aβ42/Aβ40 ratio), tauopathy (phosphorylated Tau at threonine, position 181, p-Tau181), and neuronal death (total Tau, t-Tau), others can reveal concurrent pathological events of AD, e.g., axonal injury and synaptic degeneration. Both mechanisms have gained interest as early processes because of the consistent experimental and pathological evidence describing a particular dying-back pattern of neuronal degeneration in AD, where significant impairment in synaptic function and axonal connectivity precedes neuronal death (Kanaan et al. 2013). Neurofilament light chain (NfL) is the most reliable marker of axonal damage in inflammatory and degenerative CNS conditions (Gaetani et al. 2019). CSF NfL level rises in AD already in the mild cognitive impairment (MCI-AD) stage and correlates with cognition, amyloid load, and cortical atrophy (Zetterberg et al. 2016; Dhiman et al. 2020). Similarly, synaptic degeneration is an early pathogenic event in many neurodegenerative disorders and associates with cognitive symptoms in AD (Terry et al. 1991; Blennow et al. 1996; Selkoe 2002; Lleó et al. 2019). Several pre- and post-synaptic proteins were described in the last years, but evidence of their involvement in the pathophysiology of AD is sparse (Camporesi et al. 2020). Among them, neurogranin (Ng), a dendritic protein revealing post-synaptic integrity, and two pre-synaptic proteins of the family of synucleins (beta-synuclein, β-syn, and α-synuclein, α-syn) increase in the CSF of AD patients along with cognitive impairment and disease progression (Wellington et al. 2016; Oeckl et al. 2016, 2020; Monge-Argilés et al. 2020; Halbgebauer et al. 2021).

[18]F-fluorodeoxyglucose positron emission tomography (henceforth FDG-PET) is recommended for early and differential diagnosis in patients with suspected

AD (Garibotto et al. 2017; Nobili et al. 2018a) and is acknowledged as a biomarker of downstream neurodegeneration in the research framework for AD (Jack et al. 2018) FDG-PET is a multifaceted measure of neuronal-astrocyte activity, neuropil loss, and synaptic density (Herholz 2003; Zimmer et al. 2017), much more complex than the other neurodegeneration biomarkers and thus leading to suggest a 'fourth,' independent factor in the A/T/N system, the A/T/N/F system (Ou et al. 2019). FDG-PET is also sensitive to deafferentation, diaschisis phenomena, and axonal damage (Nakashima et al. 2007; Provost et al. 2021). These processes change their relative weight throughout the AD course and contribute to the differences in topography and intensity of hypometabolism between patients and across stages (Brown et al., 2014; Mosconi, 2005; Silverman al., 2001). Within this framework, one might expect particular topographic correlations with those CSF proteins that reflect specific underlying pathological processes. Unveiling these complex relationships is even more relevant in the earliest phases of AD when understanding biomarkers changes could help to stage patients across the disease spectrum, improve phenotyping, and suggest the risk of progression to dementia.

We focused on a cohort of patients with MCI-AD to explore the brain metabolic correlates of some emerging CSF biomarkers (Ng, β-syn, α-syn, and NfL). Our goal was twofold. On the one hand, to investigate the topographic correlation of these biomarkers with brain glucose metabolism. On the other hand, to unravel whether -and to what extent- these correlating sites overlap the hypometabolic areas or regard still relatively preserved regions. This preliminary data may be helpful in terms of learning more about the value of altered CSF proteins in the prodromal phase of AD and the meaning of hypometabolism in specific areas.

2. Materials and methods

2.1 Patients

We retrospectively selected a cohort of 26 patients (16 females; age 75.4±6.6 (range 66.0-76.6); education 9.6±4.0 yrs. (range 5-17); MMSE score 26.1±1.9 (range 23-29) with typical, high-likelihood MCI-AD according to NIA-AA criteria (Albert et al. 2011b). They were extracted from the database of patients diagnosed with MCI-

AD at the memory clinic of the University of Genoa from June 2018 to January 2021. All patients had pathological values of Aβ42/Aβ40 ratio and p-Tau181, irrespective of the level of t-Tau, according to the AT(N) biomarker profile (Jack et al. 2018). Further details are in section 2.2.2.

These patients were selected because they also underwent FDG-PET during the diagnostic workup, within two months from CSF examination. At their first evaluation, they underwent neurological, general medical examinations, and an extended battery of neuropsychological tests, which defined their condition as amnestic MCI, either single- or multidomain (Petersen et al. 2009). We excluded patients with a non-amnestic presentation and performed blood tests and brain MRI to rule out non-degenerative causes of cognitive deficit. The presence of white matter hyperintensities was not an exclusion criterion if the Wahlund score was <2 in each site (Wahlund et al. 2001).

Eleven out of 26 patients converted to typical AD dementia after a mean of 1.2±0.3 years from baseline (range 0.65-1.8 years) (fast-converted), whereas the other 15 remained in the MCI stage after the last 2-year follow-up visit. We label these subjects as 'non-converted'. Because follow-up time is still limited, we are aware that most of them will probably convert to AD dementia in later years, thus virtually including only 'late' converters.

Finally, we included a healthy control (HC) group made up of 40 age-, sex- and education-matched volunteers who performed FDG-PET during previous research (20 females; age 75.6±5.0 (range 54.9-85.1); education 10.7±3.6 yrs. (range 5-17); MMSE score 28.9±1.0 (range 27-29). We checked their health condition according to clinical history and examination, with MMSE score>27, CDR=0, and a normal FDG-PET scan as established by the independent visual reading of two expert nuclear medicine physicians

All subjects gave their written consent to use anonymized data for research purposes according to the university hospital's rules for using retrospective data collected during the clinical routine. The study strictly followed the principles of the Helsinki declaration.

2.2 CSF biomarkers

2.2.1. CSF collection and pre-processing

According to the standard operating procedures, CSF samples (6–8 mL) were collected by lumbar puncture in the L3-L4 or L4-L5 interspace early in the morning (Teunissen et al. 2009). We collected CSF in sterile polypropylene tubes, centrifuged for 10 min at 4000 g at 4°C, and stored the aliquots in polypropylene tubes at –80°C until analysis to ensure long-term stability of proteins.

2.2.2. Measurement

In all patients, we measured the core CSF AD biomarkers (Aβ42, Aβ40, p-Tau181, and t-Tau) through the Lumipulse G600 II® fully automated chemiluminescent enzyme immunoassay system (Fujirebio Europe, Gent, Belgium). The standard cut-off values reported in the datasheets of the biomarker assay cartridges by the manufacturers were validated in our center as well: 0.069 for Aβ42/Aβ40 ratio, 56.5 pg/mL for p-Tau181, and 404 pg/mL for t-Tau. Samples with pathological levels of both Aβ42/Aβ40 ratio and p-Tau181, with either positive or negative t-Tau levels (n=23 and n=3, respectively), were classified as AD (A+T+(N)) (Jack et al. 2018). However, those three patients had an impaired FDG-PET so that all patients were actually A+T+N+.

In the same samples, we performed the quantitative determination in CSF of i) Ng (trunc p75) and α-syn using commercially available ELISA kits (Euroimmun, Lubeck, Germany) ii) β-syn by the recently established sandwich ELISA method, as described in detail in Halbgebauer et al. (2021) iii) NfL through Simple Plex™ Ella™ automated microfluidic platform (ProteinSimple). Concentration was in pg/ml for all these proteins. The average coefficient of variation (CV) for intra- and inter-test reproducibility was lower than 10% and 15%, respectively, for all the assays.

2.3 FDG-PET

2.3.1 FDG-PET protocol and image pre-processing

We acquired FDG-PET scans at baseline according to the European Association of Nuclear Medicine (EANM) guidelines (Varrone et al. 2009). The measured blood glucose level was less than 7.8 mmol/l after at least 6 hours of fasting. After a 10-minute rest in a quiet, dimly lit room with unplugged ears and open eyes, 185-250 MBq of [^{18}]F-FDG was injected via a venous cannula. Approximately 45 minutes after the injection, the PET scan began and lasted 15 minutes (Siemens Biograph 16 PET/CT system). We reconstructed the acquired images with an ordered subset-expectation maximization algorithm following the standard protocols used for clinical purposes and embedded in the equipment workstations. Attenuation correction was based on CT scan.

We used MATLAB and Statistical Parametric Mapping software (SPM12, Wellcome Trust Center for Neuroimaging, London, UK) for image pre-processing. PET images were normalized into a specific FDG-PET template in the MNI stereotaxic space (Della Rosa et al. 2014) and resampled to 2x2x2 mm^3 voxels, then spatially smoothed using a 10-mm isotropic Gaussian filter.

A neurologist (FM) and a nuclear medicine physician (SM) with expertise in PET analysis and interpretation individually re-valuated each image to check the quality and correctness of the pre-processing steps.

2.3.2 Voxel-based group analysis

After pre-processing, smoothed images were subjected to whole-brain voxel-wise group analyses, including:

1) comparison between MCI-AD and HC groups (two-sample t-test; nuisance: age).
2) linear correlation analysis between relative brain metabolism and CSF levels of NfL, Ng, β-syn, and α-syn, respectively, in MCI-AD patients (multiple regression analysis; nuisances: age and MMSE score).

We applied the standard 0.8 gray matter threshold masking and the default value of 50 for the grand mean scaling in all the analyses. The output was an SPM t-Map showing the statistically significant clusters using a height threshold of $p<0.001$, uncorrected for multiple comparisons at the voxel level. We considered only clusters of at least 50 voxels if statistically significant with $p<0.05$, family-wise corrected

(FWE) for multiple comparisons at the cluster level. We converted clusters coordinates using Ginger Ale (Eickhoff et al. 2009) and Talairach Client software to the Talairach's 3D coordinates system (Lancaster et al. 2000) to identify resultant cerebral areas according to the Brodmann classification.

2.3.3 Volumes of interest (VOI) analysis

The volumes of interest (VOIs) were identified using the significant clusters deriving from i) comparison between MCI-AD and HC groups and ii) correlation of each CSF biomarker level with brain metabolism. The VOI count densities in each cluster were extracted using the Marsbar toolbox implemented in SPM, then scaled to the whole brain count density in each patient.

2.4. Statistics

We compared demographic and clinical data between two subgroups of MCI-AD patients, namely fast- and non-converted. We used unpaired two-tailed T-test, or Wilcoxon rank-sum test when needed, to compare continuous data, and Fisher's exact test for categorical variables.

Furthermore, we explored differences between fast- and non-converted patients in terms of the whole-brain scaled average count densities of each of the significant VOIs (Wilcoxon rank-sum test with false discovery rate, FDR, method to account for multiple comparisons).

$p<0.05$ was considered the first level of statistical significance for all the analyses.

We computed the overlap coefficient (in percentage) to estimate the spatial interactions among the different VOIs as: (the size of the intersection of two VOIs divided by the smaller of the two VOIs) * 100.

3. Results

3.1. Comparison between patients with MCI-AD and HC

The main clinical features and biomarker values of MCI-AD patients are summarized in Table 1.

	MCI-AD (n=26)	**Late/non-converted (n=15)**	**Fast-converted (n=11)**	**p-value[#]**
Age (y)	75.4 ± 6.6	74.4 ± 4.4	76.8 ± 8.7	0.42[*]
Sex (M:F)	10:16	5:10	5:6	0.69[#]
Education	9.6±4.0	10.7 ± 3.5	8.2±4.4	0.17[*]
MMSE score	26.1±1.9	27.0±1.3	24.9 ±2.0	**0.007**[*]
Right-handed	26/26	15/15	11/11	1
Aβ42 (pg/ml)	507.5±212.3	515.5±218.9	496.5±213.0	0.90[§]
Aβ42/ Aβ40 ratio	0.043±0.01	0.041±0.010	0.046±0.009	0.28[§]
p-Tau181 (pg/ml)	113.3±42.3	113.7±46.3	112.9±38.3	0.80[§]
t-Tau (pg/ml)	718.5±253.2	731.4±275.3	700.3±231.5	0.80[§]
Ng (pg/ml)	594.8±196.1.	572.6±191.3	625.0±207.8	0.36[§]
α-syn (pg/ml)	2027.4±583.2	2015.0±626.1	2044.4±548.5	0.90[§]
β-syn (pg/ml)	836.2±331.5	864.7±366.7	861.2±294.1	0.88[§]
NfL (pg/ml)	1227.6±842.2	1044.6±631.1	1477.3±1047.3	0.33[§]

[*]p-Values for unpaired two-tailed T-test. [#]p-Values for Fischer's exact test; [§] p-values for Wilcoxon rank-sum test. Significant values are in **bold**.

Abbreviations: MCI-AD, Mild cognitive impairment due to Alzheimer's disease; F, female; M, male; MMSE, Mini Mental State Examination; Aβ42/Aβ40 ratio, amyloid Aβ1-42 normalized for amyloid Aβ1-40; pTau181, Tau phosphorylated at threonine 181; t-Tau, total Tau; Ng, neurogranin; α-syn, alpha-synuclein; β-syn, beta-synuclein; NfL, neurofilament light chain

We found three clusters (MCIAD1-VOI, MCIAD2-VOI, and MCIAD3-VOI) of significant relative hypometabolism in MCI-AD patients compared to healthy subjects. These clusters encompassed the bilateral precuneus/posterior cingulate (PC/PCC) and temporo-parietal junction, with maximal expression in the left hemisphere (Figure 1, Table 2).

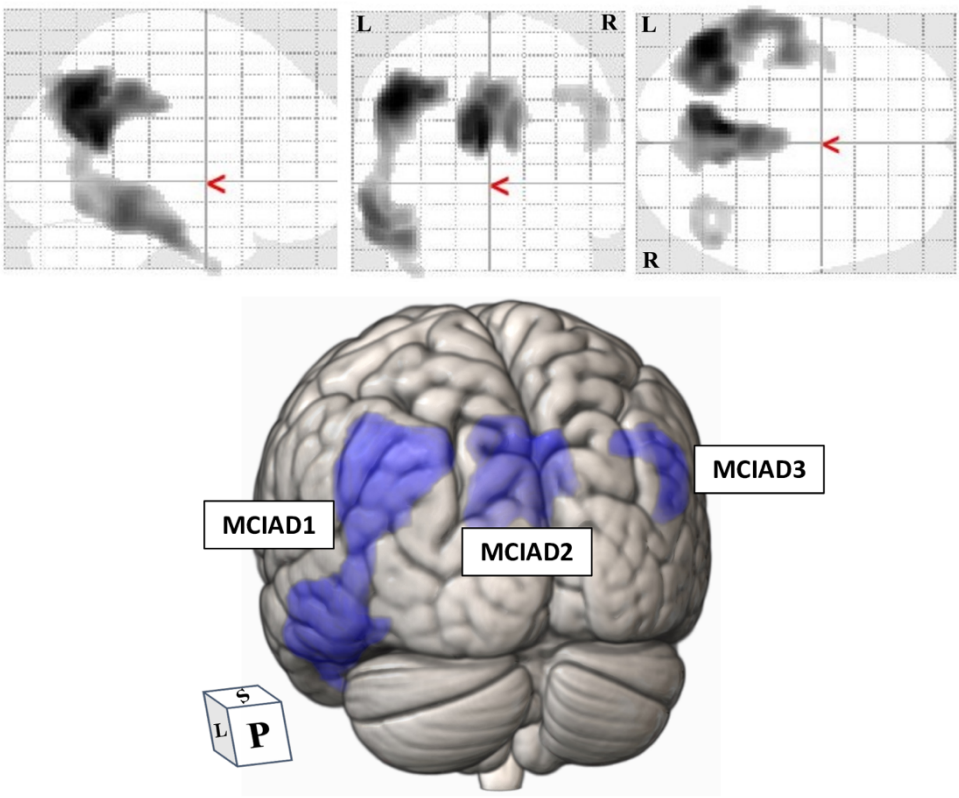

Figure 1. In the upper part, T-maps as seen from the right, back and top of the three clusters (MCIAD1, MCIAD2, and MCIAD3) from the direct comparison between MCI-AD and HC groups (height threshold of $p<0.001$, uncorrected for multiple comparisons at peak level; $p<0.05$ FWE for multiple comparisons at cluster level).

In the lower part, 3D rendering of the same clusters (shown in purple) in the MNI referential atlas (MRIcroGL software, https://www.nitrc.org/projects/mricrogl).

Abbreviations: L, left; R, right; P, posterior; S, superior

Table 2. Significant brain areas resulting from the comparisons of brain metabolism between MCI-AD and HC groups and from the correlation of CSF biomarkers with brain metabolism

Hypometabolism in MCI-AD with respect to HC group (MCIAD-VOIs)						
Cluster extent	**Cluster Significance**	**Cluster peaks coordinates**			**Cortical region (gyrus)**	**BA**
		x	**y**	**z**		
4302	p < .05 FWE corr	-46.04	-64.47	31.62	Left angular	39
(MCIAD1-VOI)		-33.09	-55.39	34.5	Left angular	39
		-56.43	-39.04	-16.59	Left inferior temporal	20
		-56.29	-19.71	-21.96	Left fusiform	20
		-56.36	-29.37	-19.28	Left inferior temporal	20
		-50.98	-58.05	-14.7	Left fusiform	37
		-51.2	-63.34	1.01	Left middle temporal	*
		-37.51	4.12	-35.6	Left middle temporal	21
		-41.24	3.97	-33.88	Left middle temporal	21
2896	p < .05 FWE corr	-6.97	-55.82	18.69	Left posterior cingulate	23
(MCIAD2-VOI)		-8.94	-67.69	24.74	Left precuneus	31
		-12.54	-65.11	17.72	Left posterior cingulate	31
		-3.44	-42.51	36.23	Left cingulate	31
		5.99	-54.03	19.08	Right posterior cingulate	23
		7.72	-58.63	27.68	Right cingulate	31
		-1.49	-30.81	31.96	Left cingulate	31
		5.85	-66.08	26.94	Right precuneus	31
541	p < .05 FWE corr	46.55	-60.87	29.93	Right middle temporal	39
(MCIAD3-VOI)		29.81	-66.9	34.48	Right precuneus	7
		46.68	-65.42	18.69	Right middle temporal	39
		39.06	-70.67	34.28	Right precuneus	39
		31.69	-57.59	35.39	Right angular	39
Correlation with neurogranin levels (Ng-VOI)						
575	p < .05 FWE corr	-16.57	-63.8	42.99	Left precuneus	7
		-7.22	-63.16	36.01	Left precuneus	7
		-5.44	-54.55	44.06	Left precuneus	7
		-8.82	-57.68	18.48	Left posterior cingulate	23
Correlation with α-synuclein levels (αsyn-VOI)						
530	p < .05 FWE corr	-7	-57.86	20.3	Left posterior cingulate	31
		-16.55	-65.49	41.03	Left precuneus	7
		-7.17	-60.94	32.61	Left precuneus	7
		-25.76	-59.68	39.63	Left superior parietal lobule	7
Correlation with β-synuclein levels (βsyn-VOI)						
573	p < .05 FWE corr	-54.92	-52.49	5.58	Left superior temporal	22
		-53.02	-50.29	2.22	Left middle temporal	22
		-44.02	-61.4	19.34	Left middle temporal	39
Correlation with neurofilament-light chain levels (NfL-VOI)						
583	p < .05 FWE corr	43.18	-42	10.04	Right superior temporal	41
		65.53	-41.07	-0.31	Right middle temporal	21
		57.98	-43.94	10.1	Right superior temporal	22
		47.08	-36.89	-3.83	Right middle temporal	37
		58.07	-61.52	-2.37	Right middle temporal	37
		63.76	-45.95	-8.01	Right middle temporal	21
		65.65	-40.19	-9.23	Right middle temporal	21
		60.23	-40.98	-20.21	Right inferior temporal	20
		58.3	-28.8	-10.08	Right middle temporal	21
		30.36	-62.89	-6.57	Right fusiform	19

Peak coordinates and cortical regions in each cluster are ordered downwards from the highest peak Z-score. In all the analyses (height threshold: uncorrected p<0.001 at peak level); clusters were considered only if they contained at least 50 voxels and were statistically significant with p <0.05, family-wise corrected (FWE corr) for multiple comparisons at cluster level.

Abbreviations: BA, Brodmann area; HC, healthy controls; MCI-AD, Mild cognitive impairment due to Alzheimer's disease; VOI, volumes of interest

3.2. Brain metabolic correlates of CSF biomarkers in MCI-AD patients

We found significant negative correlations between regional metabolic levels and CSF values of i) Ng and α-syn in similar regions of the left PC/PCC (hereafter referred to as Ng-VOI and αsyn-VOI), ii) β-syn in the left superior and middle temporal gyri (βsyn-VOI) and iii) NfL in the superior, middle, and inferior temporal gyri as well as the occipitotemporal fusiform gyrus of the right hemisphere (NfL-VOI) (Figure 2). Clusters' extent, coordinates and Brodmann areas of correlation are summarized in Table 2.

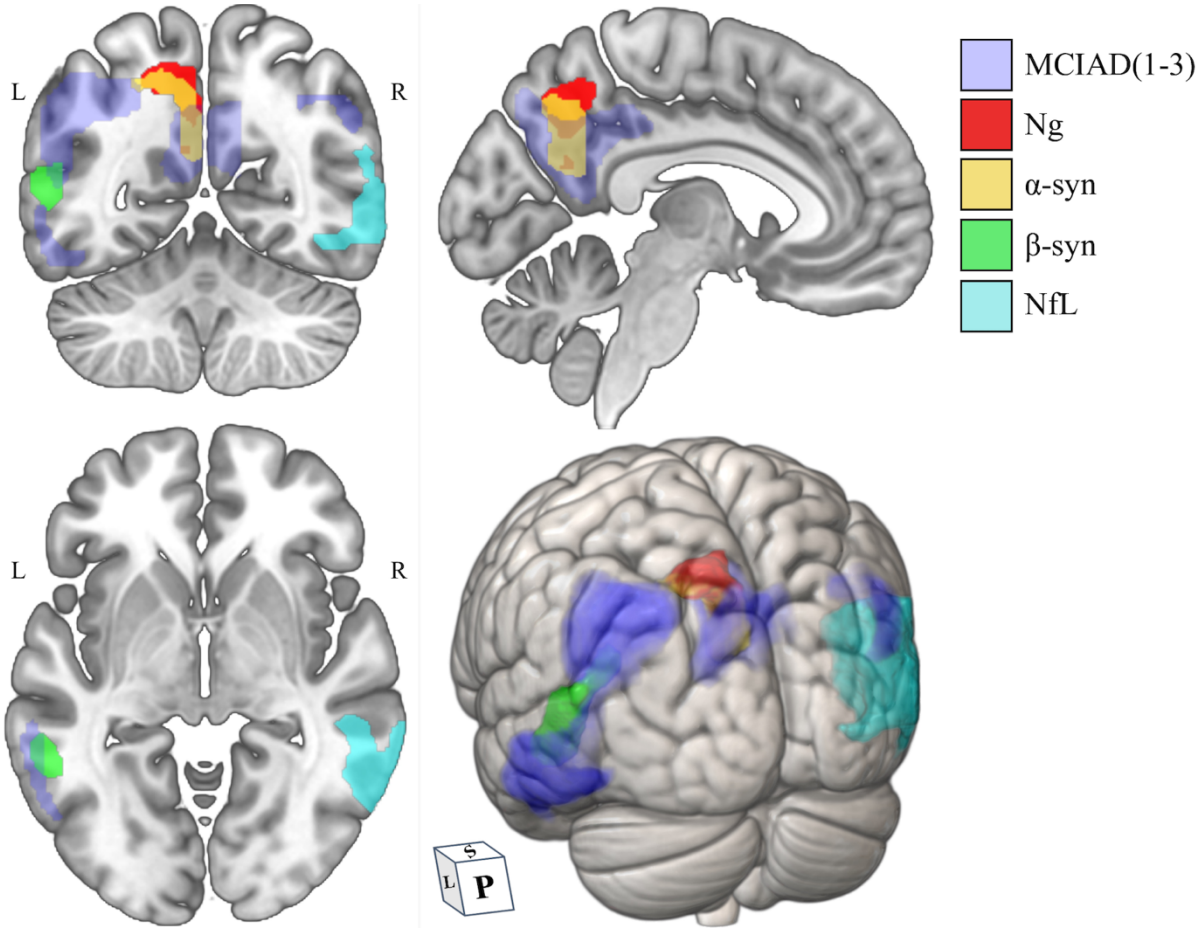

Figure 2. The significant VOIs related to neurogranin (Ng, in red), α-synuclein (α-syn, in yellow), β-synuclein (β-syn, in green), and neurofilament light chain (NfL, in light blue) are overlayed to the MCIAD clusters (already described in Figure 1, in purple). The 2D representation (axial, coronal, and sagittal cuts) and 3D rendering refer to the MNI atlas ((MRIcroGL software, https://www.nitrc.org/projects/mricrogl).

Table 3. Spatial interaction among significant VOIs expressed as overlap coefficient (%)

	MCIAD1-VOI	MCIAD2-VOI	MCIAD3-VOI	NfL-VOI	Ng-VOI	αsyn-VOI
MCIAD1-VOI						
MCIAD2-VOI	0%					
MCIAD3-VOI	0%	0%				
NfL-VOI	0%	0%	0%			
Ng-VOI	0.3%	17.4%	0%	0%		
αsyn-VOI	2.5%	54.5%	0%	0%	59.6%	
βsyn-VOI	40.3%	0%	0%	0%	0%	0%

Abbreviations: MCI-AD, Mild cognitive impairment due to Alzheimer's disease; VOIs (volumes of interest): MCIAD1-2-3, clusters (1-2-3) showing significant hypometabolism with respect to the healthy controls group; NfL, cluster significantly correlated with neurofilament light chain; Ng, cluster significantly correlated with neurogranin; **α**-syn, cluster significantly correlated with alpha-synuclein; β-syn, cluster significantly correlated with beta-synuclein; WB, whole brain

The overlap coefficients among VOIs are reported in Table 3. Briefly, the 2.5% of the αsyn-VOI was included in the MCIAD1-VOI and the 54.5% in the MCIAD2-VOI. The 0.3% of the Ng-VOI was contained in the MCIAD1-VOI and the 17.4% in the MCIAD-2VOI. The αsyn- and Ng-VOIs overlapped one another for the 59.6%. The 40.3% of the βsyn-VOI was included in the MCIAD1-VOI, whereas the NfL-VOI had no overlap with other regions.

3.3. Converted *versus* non-converted subgroup comparisons

MMSE at baseline and the whole-brain scaled average count densities of MCIAD2-VOI and MCIAD3-VOI were significantly lower in fast-converted than in non-converted patients, whereas those of the NFL-VOI only showed a trend for significance when correcting for multiple comparisons (see Table 1 and 4 for details).

Table 4. Whole brain scaled average count densities of each of the significant VOI

	MCI-AD (n=26)	Late/non-converted (n=15)	Fast-converted (n=11)	p-value[#]	q-value[*]
MCIAD1-VOI/WB	0.83±0.12	0.84±0.11	0.81±0.14	0.24	0.30
MCIAD2-VOI /WB	1.12±0.14	1.18±0.11	1.04±1.14	**0.01**	**0.047**
MCIAD3-VOI /WB	0.95±0.22	1.05±0.16	0.81±0.21	**0.003**	**0.019**
Ng-VOI /WB	0.95±0.16	0.97±0.14	0.92±0.19	0.44	0.46
α-syn-VOI /WB	0.12±0.06	1.07±0.14	1.02±0.16	0.24	0.30
β-syn-VOI /WB	0.86±0.17	0.86±0.17	0.88±0.18	0.80	0.72
NfL-VOI WB	0.74±0.16	0.81±0.09	0.66±0.2	**0.03**	0.066

[#] p-values for Wilcoxon rank-sum test; [*] FDR adjusted p-value. Significant values are in **bold**. Abbreviations: WB, whole brain, others as in Table 3.

4. Discussion

This exploratory study revealed the brain metabolic correlates of some emerging CSF biomarkers in a group of MCI-AD patients through FDG-PET voxel-based analysis. The different brain areas related to these proteins suggest they express distinct pathophysiological processes occurring in prodromal AD.

First, the metabolism in regions included in the left PC/PCC negatively correlated with CSF Ng and α-syn. Ng is a dendritic post-synaptic protein with a critical role in calmodulin-dependent memory consolidation and long-term potentiation (Hayashi 2009). Its changes in CSF closely reflect synaptic loss, cognitive decline, and neurodegeneration in AD (Portelius et al. 2015; Wellington et al. 2016; Tarawneh et al. 2016). CSF Ng values in our cohort were consistent with those previously reported with ELISA assays (Portelius et al. 2015; Sanfilippo et al. 2016; Wellington et al. 2016; Schipke et al. 2018; Willemse et al. 2018; Wang 2019), as a consequence of leakage or active secretion in the CSF after synaptic injury in AD-specific areas, such as that described in the hippocampus by pathological studies (Davidsson and Blennow 1998; Reddy et al. 2005). The CSF α-syn level similarly relates to synaptic damage and cognitive impairment in AD (Twohig and Nielsen 2019). Our results closely overlap those by Chiasserini et al. (2017) and Majbour et al. (2017) using the same ELISA assay and fit with the hypothesis of a release of α-syn from damaged neurons in prodromal AD (Twohig and Nielsen 2019). Notably, Ng is enriched primarily in the dendrites of entorhinal and hippocampal neurons (Represa et al. 1990; Guadaño-Ferraz et al. 2005) while α-syn locates in the pre-synaptic terminals of the hippocampus, amygdala, and entorhinal cortex (Twohig and Nielsen 2019). The Ng- and αsyn-VOIs largely overlapped one another (59.6%), both similarly reflecting deafferentation of the PC/PCC regions from damaged medial temporal lobe (MTL) structures. In fact, altered metabolism of remote but connected posterior areas, such as PC/PCC, is typically described in AD as a consequence of deafferentation from injured hippocampus (Matsuda 2001). This is evidenced by the overlap of hypometabolic VOIs with the αsyn-VOI (2.5% with MCIAD1 and 54.5% with MCIAD2) and Ng-VOI (0.3% with MCIAD1 and 17.4% with MCIAD2). Our results complement the findings of Portelius et al. (2015), who described a relationship between CSF Ng levels and reduced brain glucose uptake. However, they used a global PET metabolic

score obtained by averaging values of several brain regions rather than voxel-wise correlation analysis, thus a detailed topographic comparison with our results is unfeasible.

β-syn correlation sites were identified in the left superior and middle temporal areas. CSF β-syn values in our study closely overlapped with those described by Halbgebauer et al. (2021), who established the ELISA assay used in this study and confirmed the previous evidence on β-syn as a marker of pre-synaptic damage closely related to AD (Oeckl et al. 2016, 2020). Given the physiological expression of β-syn throughout cortical and subcortical brain areas (George 2001), the correlation with left lateral temporal cortex metabolism suggests an AD-specific process. This result is even more intriguing considering that a limited part of the βsyn-VOI was encompassed in the hypometabolic MCIAD1-VOI. Hence, the larger part of βsyn-VOI could express an early synaptic impairment but still not leading to overt hypometabolism in those regions. Notably, both the pre-synaptic proteins (α-syn and β-syn) were more extensively related to hypometabolic regions than post-synaptic Ng. These findings confirm the critical involvement of the pre-synaptic compartment in the dynamic of [^{18}]F -FDG in the astrocyte-neuron functional unit (Lucas et al. 2018), but also that synaptic impairment precedes neuronal damage, as suggested by previous PET studies using synaptic-specific tracers (Chen et al. 2018; Bastin et al. 2020; Mecca et al. 2020).

The correlation with areas included in the left hemisphere may not be casual, as metabolic asymmetries are frequent in early AD. Several studies highlighted the greater susceptibility of the left hemisphere to metabolic dysfunction, vascular injury, and neurodegeneration in AD (Loewenstein et al. 1989; Thompson et al. 2003; Giannakopoulos et al. 2008) and, in particular, of the left temporal lobe to altered glucose utilization due to oxidative stress (Picco et al. 2014).

The correlation of synaptic proteins with dysmetabolism in temporal areas may be read in the light of the amyloid hypothesis postulating that amyloid beta triggers a cascade harming synaptic functioning and ultimately neurons through the formation of paired helical filaments of Tau aggregates. In particular, the involvement of the lateral temporal region could reflect the progression of Alzheimer's pathology from medial to lateral superficial temporal areas (i.e., from archil- to iso- and neocortex), as

derived from neuropathological (Braak and Braak 1991; Braak et al. 2006) and Tau-PET studies (Chiotis et al. 2018).

In line with this pattern of disease progression, CSF NfL correlated negatively with lateral temporal metabolism and, albeit marginally, with the fusiform gyrus in the right hemisphere. Noteworthy, the NfL-VOI was utterly independent of the hypometabolic clusters. We can only speculate about the meaning of such intriguing finding, which might reflect an early pathological process where axonal damage - paralleled by the CSF NfL level – even precedes synaptic loss and neurodegeneration - and thus regional hypometabolism - in the less affected hemisphere.

In the same vein, previous work by our group showed that the metabolic changes of right prefrontal areas correlated with specific prospective memory tasks in MCI-AD patients, and early network disruption was significant, primarily in the interhemispheric connection (Massa et al. 2020). To note, those prefrontal regions were not hypometabolic compared to controls, like the NfL-VOI in the present study. This reinforces that progressive network disruption is critical in AD (Delbeuck et al. 2003; Agosta et al. 2011; Pagani et al. 2017) and parallels subtle metabolic changes that, at least in the earliest stages, do not result in overt hypometabolism. In agreement with the dying back hypothesis (Kanaan et al. 2013), the significant impairment in axonal connectivity precedes neurodegeneration and consequent leakage of neuronal (e.g. synaptic) proteins in CSF, hence possibly explaining why synaptic markers correlated with more damaged, dysmetabolic brain areas.

Interestingly, the whole-brain scaled mean count density of NfL-VOI was lower in fast- than non-converted patients, although such finding only showed a trend to significance ($q=0.066$) when correcting for multiple comparisons. Even if we need caution when interpreting such rather spurious result, it is concordant with a relative sparing of right hemisphere metabolism in our cohort to be associated with slower progression to dementia, as MCIAD3-VOI in the right hemisphere was significantly preserved in late converters ($q=0.019$). This is consistent with previous evidence of relatively preserved metabolism in regions within the right hemisphere (temporal areas and fusiform gyrus), acting as sites of resilience and directly associating with conversion time to dementia (Morbelli et al. 2017; Bauckneht et al. 2018).

In summary, we may put forward some hypotheses drawn by the correlations where i) Ng and α-syn reflected established hippocampal damage referring to largely deafferented and thus dysmetabolic posterior areas (PCC/PC); ii) β-syn points to the progressive synaptic and metabolic failure in left lateral temporal areas; iii) NfL relates to still relatively preserved temporal areas of the less affected (right) hemisphere, whose progressive deterioration parallels progression to dementia.

We acknowledge some limitations of this study. The relatively small sample size was due to the concomitant need to have two second-level biomarkers available (e.g., CSF and FDG-PET) and to the assessment of non-routine CSF proteins. However, the careful selection of patients with a high-likelihood AD diagnosis (Albert et al. 2011b) partially compensates for this limitation. Most patients were part of non-pharmacological clinical trials, so an atypical course or a more complex diagnosis were not the reasons they underwent both FDG-PET and CSF analysis. Furthermore, we adjusted all our analyses for age to limit its influence on CSF biomarker level, primarily NfL (Bridel et al. 2019). We included healthy subjects only to compare their brain metabolism with that of MCI-AD patients but not to assess the metabolic correlates of CSF biomarkers. The use of both biomarkers (i.e., CSF and FDG-PET) is rare in the healthy, even when included in research protocols. Without a control group for CSF biomarkers, we referenced published works that used the same assays and found similar levels in CSF. Moreover, the relatively small sample of patients led us to use a univariate analysis - where the different proteins were independently correlated with brain metabolism - rather than a multivariate one that might more appropriately estimate the relative contribution of each protein to the total variance. For the same reason, the correction for multiple comparisons limited the significance of the NfL-VOI comparison between converted patients and those not converted yet, thus suggesting that the prognostic value of NfL metabolic correlates should be cautiously interpreted and additional data are needed.

Given such limitations, our preliminary findings should be considered explorative for the MCI-AD condition with a typical amnestic presentation and need to be replicated in larger samples with a prospective design and more conservative statistical thresholds.

In conclusion, by providing evidence for the metabolic correlates of several process-specific CSF biomarkers, the present results shed light on their pathophysiological role in prodromal AD. These findings complement the valuable information on the distribution of hypometabolism related to neuronal loss, which differs from the metabolic changes reflecting synaptic loss or axonal damage. Further studies including also pre-symptomatic and demented subjects are worthful to assess the dynamic changes of CSF proteins and brain metabolism across different AD stages. This would give insight into the pathological processes occurring at different times in distinct brain regions and the resilience mechanisms that counteract them.

5. Discussion and concluding remarks

Revealing the complex interactions and assessing potential integration between biomarkers is essential, especially in the early stages of AD, when biomarker alterations may serve to stage patients throughout the disease spectrum, improve phenotyping, and indicate the likelihood of progression to dementia.

In this research, the integration of [^{18}F]-FDG-PET and CSF biomarkers, two of the most used biomarkers in centers focused on neurocognitive disorders, enabled us to collect evidence on their analytical and diagnostic performance when used in a step-wise fashion. As part of the ongoing endeavor to create a common diagnostic chart for the precise and cost-effective use of biomarkers in neurocognitive diseases with neurodegenerative origin (Festari et al. 2022), these data gain further significance.

Additionally, by combining semiquantitative [^{18}F]-FDG-PET and CSF data, we were able to identify precise topographic correlations between metabolic values and CSF proteins that indicated distinct underlying disease processes. These findings add to the knowledge regarding the distribution of hypometabolism linked to neuronal loss, which is distinct from metabolic changes reflecting synaptic or axonal injury, and provide an indirect insight of the pathological processes taking place at various times in different parts of the brain.

These results will be expanded into bigger cohorts in future research, which will also integrate additional newly discovered synaptopathy-expressing proteins for diagnostic and prognostic purposes.

6. Appendix

6.1. Publications during PhD

1. Benedetti L, Garnero M, Demichelis C, Grandis M, Briani C, Beltramini S, Bellucci M, Prada V, **Massa F**, Gastaldi M, Schenone A, Franciotta D. Outcomes after single-cycle rituximab monotherapy in patients with anti-MAG polyneuropathy: A bi-center experience with an average follow-up of 11 years. ***J Neuroimmunol.*** 2019 Oct 15;337:577081

2. Prada V, **Massa F**, Salerno A, Fregosi D, Beronio A, Serrati C, Mannironi A, Mancardi G, Schenone A, Benedetti L. Importance of intensive and prolonged rehabilitative treatment on the Guillain-Barrè syndrome long-term outcome: a retrospective study. ***Neurol Sci***. 2020 Feb;41(2):321-327

3. **Massa F,** Farotti L, Eusebi P, Capello E, Dottorini ME, Tranfaglia C, Bauckneht M, Morbelli S, Nobili F, Parnetti L. Reciprocal incremental value of 18F-FDG-PET and cerebrospinal fluid biomarkers in Mild Cognitive Impairment patients suspected for Alzheimer's disease and inconclusive first biomarker. ***J Alzheimers Dis.*** 2019;72(4):1193–1207

4. Gastaldi M, Mariotto S, Giannoccaro MP, Iorio R, Zoccarato M, Nosadini M, Benedetti L, Casagrande S, Di Filippo M, Valeriani M, Ricci S, Bova S, Arbasino C, Mauri M, Versino M, Vigevano F, Papetti L, Romoli M, Lapucci C, **Massa F**, Sartori S, Zuliani L, Barilaro A, De Gaspari P, Spagni G, Evoli A, Liguori R, Ferrari S, Marchioni E, Giometto B, Massacesi L, Franciotta D. Subgroup comparison according to clinical phenotype and serostatus in autoimmune encephalitis: a multicenter retrospective study. ***Eur J Neurol.*** 2020; 27:633-643

5. Orso B, Mattei C, Arnaldi D, **Massa F**, Serafini G, Plantone D, Doglione E, Grafman J, Nobili F, Pardini M. Clinical and MRI predictors of conversion from Mild Behavioural Impairment to dementia. ***Am J Geriatr Psych.*** 2020;28:755−76

6. Arnaldi D, Donniaquio A, Mattioli P, **Massa F**, Grazzini M, Meli R, Filippi L, Grisanti S, Famà F, Terzaghi M, Girtler N, Brugnolo A, Doglione E, Pardini M, Villani F, Nobili F. Epilepsy in Neurodegenerative Dementias: A Clinical, Epidemiological, and EEG Study. ***J Alzheimers Dis***. 2020;74(3):865-874

7. Arnaldi D, Chincarini A, De Carli F, Famà F, Girtler N, Brugnolo A, Pardini M, **Massa F**, Meli R, Schenone C, Bauckneht M, Morbelli S, Nobili F. The fate of patients with REM sleep behavior disorder and Mild Cognitive Impairment. ***Sleep Medicine***, 2021;79:205-210

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