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ADVANCED COGNITIVE, MOLECULAR AND STRUCTURAL BIOMARKERS IN PRECLINICAL AND PRODROMAL DEMENTIA PHASES

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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.

All the results presented here were obtained by myself, except for:

- **MRI data analysis included in Study 1 (Role of imaging biomarkers in the classification of MCI condition as defined by the NIA-AA criteria. Preliminary data analysis) was performed by the Laboratory of Neuroinformatics, IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy.**
- **Data collection regarding cognitive, neuropsychological and neuropsychiatric evaluations included in Study 3 (Brain metabolism and amyloid load in individuals with subjective cognitive decline or pre-Mild Cognitive Impairment) was performed by the Laboratory of Neuropsychiatry, IRCCS Santa Lucia Foundation, Rome, Italy.**

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Dedication

Some people are vital in my personal life and deserve special thanks for how close they are to me, not just in the last three years. The last three years have been filled with a bunch of things. Intense work. Suffering from isolation. The abrupt discovery of physical fragility. Worries about the distance from my loved ones. But also, unconditional love (mum and dad). Support that goes beyond the geographical distance (MT, AM, ST, FA, AT, MF, CT, CG). Relationships temporarily paused for stupid reasons but impossible to break, which will be resumed closer than before (ST). The love, grown in everyday life, such intense daily life to be gorgeous (FDM). Great moving of stuff and feelings, possible only with the help of precious new family people (IDM, MDM, CG). Close ties with other tiny living beings in fifty square meters (F, C, F). Unbreakable old and new friendship (SP, AN, EV). New acquaintances and rediscovered passions (BC). Finally, three years full of changes and surprises, love for what I do, and what I want to keep doing. Love to convey to the youngest darlings the little I know, and to continue learning from them (JT, GT, DF, DF).

I dedicate this thesis to my family, my parents, my sisters, my brothers, my nephews. To Fabiola, the person I love. To my beloved dogs. Thanks for your support and unconditional care. I love you all.

"It doesn't matter what you do, he said, so long as you change something from the way it was before you touched it into something that's like you after you take your hands away. The difference between the man who just cuts lawns and a real gardener is in the touching, he said. The lawn-cutter might just as well not have been there at all; the gardener will be there a lifetime." (Ray Bradbury, Fahrenheit 451)

"Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at." (Stephen Hawking)

LIST OF STUDIES

PUBLISHED STUDIES

Tondo G., et al., TAM Receptor Pathways at the Crossroads of Neuroinflammation and Neurodegeneration. Dis Markers. 2019. Review. (Tondo *et al*, 2019)

Tondo G., et al., The combined effects of microglia activation and brain glucose hypometabolism in early-onset Alzheimer's disease. Alzheimers Res Ther. 2020. Original Article. (Tondo *et al*, 2020b)

Tondo G., et al., Biomarker-based stability in limbic-predominant amnestic mild cognitive impairment. Eur J Neurol. 2021. Original Article. (Tondo *et al*, 2020a)

Perani D., et al., PET Neuroimaging in Dementia Conditions. PET SPECT Neurol 2020. Review. (Perani *et al*, 2020)

Tondo G., et al., Brain Metabolism and Microglia Activation in Mild Cognitive Impairment: A Combined [^{18}F]FDG and [^{11}C]-(R)-PK11195 PET Study. J Alzheimers Dis. 2021. Original Article. (Tondo *et al*, 2021)

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ABSTRACT

Dementia is a global epidemic affecting about 47 million people in 2015, reaching 135 million in 2050. This doctoral dissertation aims to explore the *in vivo* role of PET imaging along the dementia continuum, from the preclinical to the prodromal and to the overt dementia phase, to provide a comprehensive picture of the mechanisms underlying neurodegeneration.

Evidence in individuals with genetic risk to develop Alzheimer's dementia suggests that the pathophysiological process leading to neurodegeneration starts years before the clinical diagnosis of dementia. For this reason, it is crucial to investigate biomarkers at the earliest possible stage of dementia. Mild Cognitive Impairment, pre-Mild Cognitive Impairment, and Subjective Cognitive Decline represent conditions with an increased risk of developing Alzheimer's disease and other dementias, but some individuals will remain stable over time, and others will return to normal cognition. To correctly classify these subjects has significant therapeutic and prognostic repercussions. We investigated the diagnostic and prognostic role of [^{18}F]FDG-PET in identifying subjects at risk for developing dementia and individuals with a favorable prognosis. We coupled brain metabolism information with *in vivo* pathological, structural, cerebrospinal fluid, and neuropsychological and neuropsychiatric data to improve diagnostic classification and predictive accuracy. [^{18}F]FDG-PET, amyloid-PET, and Magnetic Resonance imaging analyses were performed using validated and standardized procedures, which provided reliable and trustworthy results. We also explored the role of *in vivo* neuroinflammation in different stages of neurodegeneration, including Alzheimer's disease dementia and Mild Cognitive Impairment. Neuroinflammation is a potential driver of neurodegenerative changes in several conditions, but its beneficial or detrimental role depends on the disease stage.

Overall, our results confirm that the preclinical and prodromal stages of neurodegenerative dementias are heterogeneous states, and biomarkers assessment helps to classify or exclude individuals along the trajectory of neurodegeneration. [^{18}F]FDG-PET revealed its crucial role in detecting or excluding early signs of neurodegeneration, and this would be very much contributing to the screening of candidates in clinical trials.

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ACRONYMS AND ABBREVIATIONS

^{11}C, Carbon-11

^{18}F, fluorine-18

AD, Alzheimer's Disease

ADNI, Alzheimer's Disease Neuroimaging Initiative

ALS, amyotrophic lateral sclerosis

ALSFRSr, amyotrophic lateral sclerosis Functional Rating Scale-Revised

ANOVA, one-way analysis of variance

APOE, apolipoprotein E

APP, amyloid precursor protein

AQP, Aquaporin

ATP, Adenosine triphosphate

BBB, Blood-Brain Barrier

BOLD, blood oxygen level-dependent

BvFTD, behavioral variant of Frontotemporal dementia

C, Complement protein

CBD, corticobasal degeneration

CBS, corticobasal syndrome

CCA, cerebral amyloid angiopathy

CDR, Clinical Dementia Rating scale

CNS, central nervous system

CSF, cerebrospinal fluid

DAMPs, damage-associated molecular patterns

DED, Deuterium-l-deprenyl

DLB, dementia with Lewy bodies

DSM, Diagnostic and Statistical Manual of Mental Disorders

EOAD, early-onset Alzheimer's disease

FAQ, Functional Assessment Questionnaire

FDG, fluorodeoxyglucose

FCSRT, Free and Cued Selective Reminding Test

FTD, frontotemporal dementia

FTLD, frontotemporal lobe degeneration

FUS, fused in sarcoma

Gas6, Growth Arrest Specific 6

IADL, Instrumental Activities of Daily Living

IL, Interleukin

IFN, interferon

IWG, International Working Group

JAK, Janus Kinase

LATE, limbic-predominant age-related TDP-43 encephalopathy

LOAD, late-onset Alzheimer's disease

LPS, lipopolysaccharide

MAO-B, Monoamine oxidase-B

MAPT, microtubule-associated protein tau

MCI, mild cognitive impairment

MMSE, Mini Mental State Examination

MND, motor neuron disease

MNI, Montreal Neurological Institute

MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRI, magnetic resonance imaging

MSA, multiple system atrophy

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NIA-AA, National Institute on Aging and Alzheimer's Association

NINCDS-ADRDA, National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association

NK, Natural Killer

NLR, nuclear oligomerization domain-like receptors

PAMPs, pathogen-associated molecular patterns

PART, primary age-related tauopathy

PCA, posterior cortical atrophy

PD, Parkinson's disease

PET, Positron Emission Tomography

PiB, Pittsburgh compound B

PPA, primary progressive aphasia

PSEN1, presenilin 1

PSEN2, presenilin 2

PSP, progressive supranuclear palsy

P-tau, hyperphosphorylated-tau

RAGEs, receptors for advanced glycation end products

SCD, Subjective Cognitive Decline

SD, semantic dementia

SPM, Statistical Parametric Mapping

SOD1, superoxide dismutase 1

STAT, activator of transcription

SUVr, Standard Uptake Value ratio

TAMs, Tyro3-Axl-Mertk receptors

TDP-43, TAR DNA-binding protein 43

TGF, Transforming Growth Factor

Th, T helper lymphocyte

TLR, Toll-like receptor

TNF, Tumor Necrosis Factor

TSPO, 18 kDa translocator protein

Tregs, T regulatory cells

TREM, triggering receptor expressed on myeloid cells

T-tau, total-tau

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INTRODUCTION

The Alzheimer's disease spectrum

Alzheimer's disease (AD) is a neurodegenerative disorder progressively leading to cognitive decline, behavioral disturbances, and functional dependency. AD is the most common neurodegenerative disease, the most frequent etiology of dementia, and among the ten most common causes of death globally (Rana *et al*, 2021). Its prevalence is continuously increasing due to the progressive aging of the world's population since advanced age represents the greatest risk factor for AD. For these reasons, AD is now considered a healthcare burden of epidemic proportions, still lacking an effective disease-modifying treatment (Crous-Bou *et al*, 2017). Furthermore, the irreversible progression of cognitive and behavioral disability produces a devasting socio-economic impact. An Italian cost-of-illness study estimated that the social burden of AD, considering public, patient, and caregiver costs reached about 20.000 euros per year, which were managed predominantly by AD patient families (Chiatti *et al*, 2015). The principal costs related to the management of people with dementia are associated with institutionalization, which represents an unrecoverable choice in about 50% of dementia cases after five years of disease and in up to 90% of cases after eight years of disease (Luppa *et al*, 2010). Risks prevention, early diagnosis, and accurate prognostic stratification are essential to contrast AD's massive healthcare, social and economic impact. To improve diagnostic workup and risk prevention strategies series of criteria for AD have been perfected in the last twenty years, primarily based on the pathological identification of amyloid-β pathology and tau neurofibrillary tangles. To date, the research on disease-modifying therapies for AD has focused primarily on lowering brain amyloid-β levels, but without reaching significant clinical benefit. However, due to the closer relationship between neurofibrillary tangles and cognitive impairment, targeting tau pathology might be clinically more relevant (Giacobini & Gold, 2013). Identifying neurodegenerative abnormalities at the earliest possible stage is even more compelling. The biological definition of AD motivated the recognition of a long pre-dementia stage, preceding the clinical appearance of symptoms, representing a potential fruitful therapeutic target (Dubois *et al*, 2016). In this pre-dementia stage, while pathological alteration can be

already detectable, irreversible neurodegenerative processes may not yet occur, offering the possibility to change the disease course effectively.

The definition of Alzheimer's disease

At the beginning of the XX century, the psychiatrist and neuropathologist Alois Alzheimer, working in Frankfurt, met and subsequently studied a patient, Auguste Dieter, suffering from rapid cognitive decline, memory impairment, and personality changes. After her death, Alzheimer performed a detailed autoptic analysis of the brain. The results were described in 1906 at the meeting of Tubingen and later published in a report in 1911. The pathological examination revealed diffused plaques and tangles in the brain and extensive neuronal loss (Alzheimer, 1911). This Alzheimer's first description paved the way for the subsequent extensive characterization of the clinical entity, named AD, as Emil Kraepelin defined it in 1909. The association between pathological brain changes and the severity of dementia was reported many years later (Blessed *et al*, 1968), as well as the identification of the amyloid protein as the main constituent of the senile plaques and the tau as the principal protein in tau tangles (Glenner & Wong, 1984; Leoni *et al*, 2017). The National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) workgroup proposed, in 1984, the first set of structured diagnostic criteria for AD (McKhann *et al*, 1984). According to this classification, "probable" AD dementia was defined as an insidious onset with progressive memory impairment and other cognitive decline, affecting autonomy in daily life activities. Other causes of dementia should have been excluded. At this stage, no laboratory tests could aid clinical diagnosis of probable AD, mainly based on medical history and neurological and neuropsychological examination. The "definite" diagnosis of AD dementia was confined to those cases with autopsy confirmation of neuropathological alterations characteristic of AD, namely the presence of neuritic plaques, neurofibrillary tangles, and neuronal loss. Therefore, the diagnostic criteria proposed a clinical-pathological definition of AD, limiting the clinical definition to the probable diagnosis (Knopman *et al*, 2019).

The revised version of the Diagnostic and Statistical Manual of Mental Disorders (DSM), third edition, published in 1987, proposed a set of criteria for diagnosing

dementia of Alzheimer's type in line with the NINCDS-ADRDA definition. The classification included patients with memory impairment associated with other cognitive domains decline or personality changes, significantly affecting working or social activities and relationships (American Psychiatric Association, 1987).

The conceptualization of AD as a clinical-biological entity elaborated in the eighties was widely accepted and applied to clinical activity for over 30 years (Petersen, 2018). However, several diagnostic problems quickly emerged, pushing physicians and researchers to operate new classifications. By evaluating the diagnostic accuracy of the clinical diagnosis of AD, using the autoptic confirmation as the gold standard, the mean sensitivity was about 81% and the mean specificity of about 70%, suggesting that non-AD dementia shares with AD dementia many common features (Knopman *et al*, 2001). Early neuropathological reports showed that pure AD pathological changes, including neuritic plaques and neurofibrillary tangles, represented only a part of the clinically diagnosed cases of AD. Most cases showed the concomitant presence of additional pathology, indeed (Bowler *et al*, 1998). In addition, most aged people showed many distinct brain pathologies, including hippocampal sclerosis and cerebrovascular disease, associated with aging and not necessarily linked to AD and dementia (Nelson *et al*, 2011).

In 2000 the DSM-IV was revised, and diagnostic criteria for dementia of AD type still required the presence of a memory impairment associated with the decline in at least one cognitive domain, interfering with autonomy in activities of daily living and social functioning (American Psychiatric Association, 2000).

Seven years later, in 2007, the NINCDS-ADRDA criteria were firstly reviewed by the International Working Group (IWG) (Dubois *et al*, 2007). For the first time, *in vivo* biomarkers were proposed to support diagnosis and characterize the clinical-biological entity of AD in the predementia stage when symptoms do not configure the clear picture of overt dementia. The "prodromal AD" stage included a wide range of described entities, such as age-related memory impairment and cognitive decline, mild neurocognitive disorder, and mild cognitive impairment (MCI). These terms identified individuals with cognitive decline but normal functional abilities and global cognitive status, showing a higher risk than normal cognitive age-matched individuals to develop AD dementia, but not invariably progressing to dementia (Dubois *et al*, 2007). The need to identify and classify these subjects arose from the possibility that potential therapeutic strategies,

aiming at blocking the pathological cascade leading to neurodegeneration and dementia, would have been effective only in an early, not yet irreversible phase. However, the heterogeneity of the neuropathological substrate of the condition and consequent different prognostic evolution was evident at this stage. Most MCI showed AD neuropathological changes at the pathological exam, while some MCI manifested other brain abnormalities, including argyrophilic grain disease, hippocampal sclerosis, and vascular lesions (Petersen *et al*, 2006). Therefore, different clinical phenotypes were proposed, and the amnestic MCI was assumed to represent the prodromal phase of AD dementia (Petersen, 2004). The IWG revision of the NINCDS-ADRDA criteria aimed to identify individuals in the prodromal phase, improve AD diagnostic specificity, improve diagnosis of non-AD dementia, and characterize different AD phenotypes. The previous classification exalted the amnestic presentation of AD, which is the most frequent but not the only possible presentation, considering that AD can manifest with prominent language, executive, or visual-perceptive disturbances. In addition, the revision added the support of biomarkers, including both markers for neurodegeneration and pathology. The detection of medial temporal lobe atrophy on magnetic resonance imaging (MRI), the presence of abnormal cerebrospinal fluid (CSF) levels of amyloid-β or tau proteins, the identification of brain metabolism alterations with the fluorine-18 fluorodeoxyglucose ([^{18}F]FDG)-Positron Emission Tomography (PET), added necessary support to the clinical diagnosis of AD (Dubois *et al*, 2007). The introduction of *in vivo* biomarkers for AD pathology represented a crucial step in AD definition. CSF detection of low levels of amyloid-β and high levels of total-tau (t-tau) and hyperphosphorylated-tau (p-tau) were recognized as measurements of brain amyloidosis, neurodegeneration, and tau tangles deposition (Mattsson *et al*, 2009). The PET tracer carbon-11 radioligand Pittsburgh compound B ([^{11}C]PiB) showed to be able to identify cortical deposition of amyloid-β plaques in living persons (Klunk *et al*, 2004). Very quickly, amyloid-β markers offered the possibility to obtain the AD diagnosis during life, allowing to extend the clinical suspect and the prognostic considerations to the very early stage of the disease (Knopman *et al*, 2019).

In 2010, the IWG proposed a new classification, defining AD as a spectrum, including patients with overt dementia and individuals with mild or no symptoms (Dubois *et al*, 2010). The AD spectrum included asymptomatic people at risk for developing dementia,

specifically showing biomarker's evidence of AD pathology, and pre-symptomatic people carrying monogenic AD mutations, who inevitably will develop AD dementia. In this revision, a clear distinction was proposed between the clinical diagnosis and disease pathology, considering that the evidence of AD pathological changes not necessarily coincides with the stage of AD dementia. In addition, the term "atypical AD" was proposed to identify patients with non-amnestic onset, namely the logopenic variant of primary progressive aphasia (PPA) (language), the posterior cortical atrophy (PCA) (visual), and the frontal AD variant (dysexecutive/behavioral). Finally, the term "mixed AD" was introduced to indicate patients with evidence of AD pathology plus a second pathobiological process (Dubois *et al*, 2010). The subsequent revision of the IWG criteria in 2014 detailed all these concepts, proposing the diagnosis of AD as a combination of clinical (amnestic dementia) and biological (biomarkers) evidence (Dubois *et al*, 2014). Amnestic dementia represented the typical AD. The biomarkers were classified in markers of amyloid pathology and downstream topographical markers, referring to markers of neuronal injury, including CSF t-tau, brain hypometabolism as detected by [^{18}F]FDG-PET, neuronal loss as shown by MRI hippocampal atrophy. The definitions of prodromal AD (MCI without substantial impairment in daily life functioning) and asymptomatic at risk for AD completed the AD spectrum definition with the prodromal and preclinical phases (Dubois *et al*, 2014).

With the development and increased availability of biomarkers for AD, the National Institute on Aging and Alzheimer's Association (NIA-AA) proposed a theoretical model with subsequent revisions aiming to define the pathological changes of AD *in vivo* and antedating the development of clinical manifestations (Jack Jr *et al*, 2010a). Following the amyloid cascade hypothesis, this model proposed that amyloid-β deposition precede tau tangle formation and subsequent neurodegenerative changes, which can be detected by brain MRI and [^{18}F]FDG-PET. Thus, the cognitive decline appears later over the disease stage, preceded by pathological and molecular alterations, and manifests initially with MCI and then with AD dementia. Adding tau pathology as a primary event in the cascade, this model has been further updated, specifying that amyloid-β and tau deposition may occur independently in AD but inevitably preceding neurodegeneration markers and symptoms onset (Jack Jr *et al*, 2013). This scheme was confirmed by studies in autosomal dominant AD, which is a rare inherited form of AD due to mutations in one

of three genes - amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), or presenilin 2 (*PSEN2*), representing less than 1% of AD cases and manifesting with early-onset dementia, generally at 30-50 years (Bateman *et al*, 2011). In genetic AD patients, CSF amyloid-β levels decline more than two decades before the expected onset of the symptoms, while cortical amyloid deposition as visualized by amyloid-PET can be detected later. Increased tau CSF levels, brain atrophy, and brain hypometabolism can be identified ten-to-fifteen years before the symptom's onset, while global cognitive impairment occurs within five years to the onset of symptoms (Bateman *et al*, 2012). **Figure 1** represents the chronological alteration of biomarkers in inherited AD. In line with this evidence, in 2011, the NIA-AA formulated a diagnostic scheme based on the amyloid cascade hypothesis and the availability of biomarkers, proposing the conceptualization of the clinical continuum of AD (McKhann *et al*, 2011). The proposed classification identifies three phases: 1) the preclinical AD, where clinically unimpaired individuals show brain pathological changes, including amyloid deposition and neurodegeneration; 2) the prodromal AD, including MCI individuals, with a subtle cognitive decline but without impact on daily activity functioning; 3) the overt dementia phase (Jack Jr *et al*, 2011; McKhann *et al*, 2011; Albert *et al*, 2011; Sperling *et al*, 2011).

According to the NIA-AA criteria, AD is classified as a continuum in which biomarkers add certainty to the clinicopathological substrate. The biomarkers are used to identify the presence of amyloid and neurodegeneration changes, aiming to *in vivo* confirm that the clinical syndrome is due to AD pathology. They are critical in MCI individuals, where pathology influences prognosis and the evidence of abnormal brain amyloid load and neurodegeneration changes entails a diagnosis of MCI due to AD with a high likelihood (Albert *et al*, 2011). The definition of an AD continuum based on pathological and molecular evidence further sustained the view of AD as a biological construct.

In the same years, the DSM-V criteria rekindled the debate, highlighting the split between the clinical diagnosis of cognitive impairment and its etiology. The classification system employed “major neurocognitive disorder” to define the dementia stage and “mild neurocognitive disorder” to classify MCI individuals independently from the etiology. After the delineation of the clinical diagnosis, biomarkers were proposed to clarify the etiological diagnosis. In this classification, the first step, which is the clinical diagnosis,

allows the identification of all the different forms of dementia, and further characterization is recommended for prognostic purposes (American Psychiatric Association, 2013).

The advent of tau-PET imaging, allowing to visualize *in vivo* brain tau pathology, further aided the possibility to detect AD pathology in living individuals. The NIA-AA developed and then encouraged the utilization of a biomarker-based classification system, the ATN. The ATN system recognizes three biomarkers groups based on the pathological and molecular processes that they can detect. "A" stands for amyloid and identifies the presence of brain amyloid plaques as detected by amyloid-PET or underlined by low CSF levels of amyloid-β. "T" stands for tau, referring to the evidence of increased p-tau levels in the CSF or increased tau tracer binding at the tau-PET imaging. "N" stands for neurodegeneration and indicates the neuroimaging evidence of neurodegenerative changes, both atrophy testified by MRI and hypometabolism detected by [^{18}F]FDG-PET, or increased levels of CSF t-tau (Jack *et al*, 2016). Following the ATN scheme, in 2018, the NIA-AA proposed a structured research framework characterizing AD as a biological entity, with definitions applicable both at the symptomatic and at the pre-symptomatic stage (Jack Jr *et al*, 2018a). According to this framework, the term "AD" should be restricted to individuals with *in vivo* evidence of amyloid-β and tau pathology (positive biomarkers), regardless of the subject's clinical status. Abnormal amyloid-β without concomitant tau pathology does not configure AD, even if it may represent an AD prodromal stage. The framework classifies patients with dementia in different categories: a) patients with AD dementia if they show both amyloid and tau positivity, regardless of the presence of neurodegeneration; b) AD pathological change with dementia and AD with concomitant non-AD pathology in case of amyloid positivity, c) tau negativity, and respectively with absent or evident neurodegeneration; d) non-AD pathologic change with dementia in case of normal amyloid and tau or neurodegeneration positivity; e) normal AD biomarkers in case of absence of amyloid and tau pathology and neurodegeneration (Jack Jr *et al*, 2018a). This framework allows to separate the biological definition of AD from the clinical phenotype, thus extending the same classifications (AD, AD pathologic change, AD pathologic change with concomitant non-AD pathology, non-AD pathologic change, normal biomarkers) at the MCI condition and at the cognitively unimpaired individuals (see **Figure 2**). However, while the conceptual

framework aimed to define the AD continuum as a biological entity, the clinical spectrum was not neglected. In order to aid the design of clinical trials, a numerical staging system was also elaborated. This classification system identified different stages for individuals along the AD continuum, namely showing AD pathological changes: 1) cognitively unimpaired individuals; 2) subjects with a subtle sign of cognitive impairment but normal global cognition, including individuals with subjective cognitive impairment without objective impaired performances on neuropsychological evaluation; 3) MCI; 4) mild dementia; 5) moderate dementia; 6) severe dementia (Jack Jr *et al*, 2018a). The ATN classification system again supports the clinical staging, since abnormalities in all biomarkers testify to a more advanced pathological stage. The rate of cognitive impairment in cognitively normal and impaired individuals showing biomarker alterations is significantly higher when compared with individuals with a normal biomarkers profile, confirming the prognostic value of the ATN system, the accuracy in staging individuals in the AD continuum, and its usefulness in screening participants for clinical trials (Caroli *et al*, 2015; Mormino *et al*, 2014; Allegri *et al*, 2020; Ebenau *et al*, 2020).

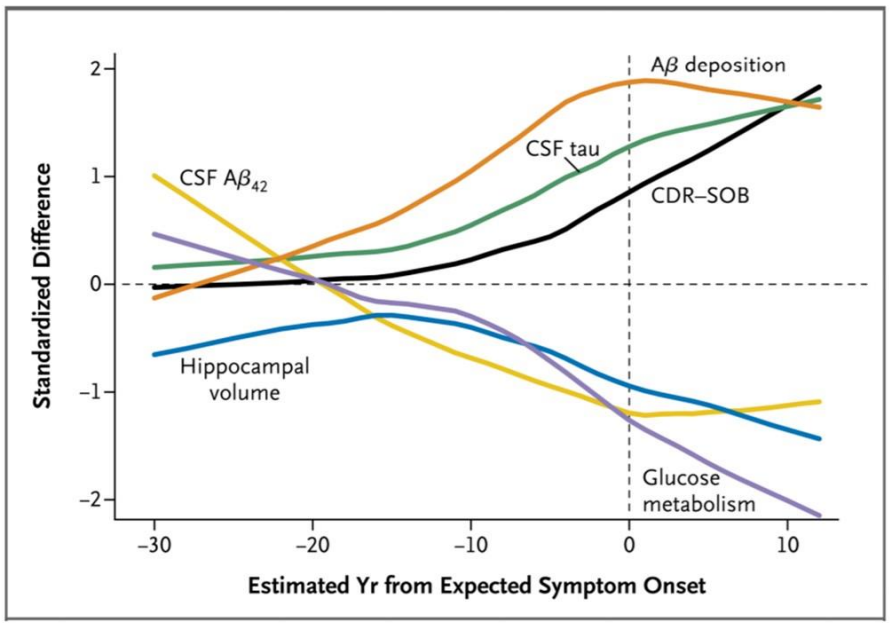


Figure 1. Chronological alteration of clinical, cognitive, structural, metabolic ad biochemical changes in individuals with genetic Alzheimer's disease in relation to the expected onset of symptoms (source: Bateman *et al.*, 2012).

		Cognitive stage		
		Cognitively Unimpaired	**Mild Cognitive Impairment**	**Dementia**
Biomarker Profile	$A^- T^- (N)^-$	normal AD biomarkers. cognitively unimpaired	normal AD biomarkers with MCI	normal AD biomarkers with dementia
	$A^+ T (N)$	Preclinical Alzheimer's pathologic change	Alzheimer's pathologic change with MCI	Alzheimer's pathologic change with dementia
	$A^+ T^+ (N)^-$	Preclinical Alzheimer's disease	Alzheimer's disease with MCI(Prodromal AD)	Alzheimer's disease with dementia
	$A^+ T^+ (N)^+$			
	$A^+ T (N)^+$	Alzheimer's and concomitant suspected non Alzheimer's pathologic change, cognitively unimpaired	Alzheimer's and concomitant suspected non Alzheimer's pathologic change with MCI	Alzheimer's and concomitant suspected non Alzheimer's pathologic change with dementia
	$A^- T^+ (N)^-$	non-Alzheimer's pathologic change, cognitively unimpaired	non-Alzheimer's pathologic change with MCI	non-Alzheimer's pathologic change with dementia
	$A^- T^- (N)^+$			
	$A^- T^+ (W^+$			

Figure 2. Descriptive classification of individuals along the dementia continuum, combining the syndromic cognitive staging with biomarker alterations (source: Jack *et al.*, 2018a).

The prodromal and preclinical phases of Alzheimer's disease

AD dementia still lacks curative therapy, and a discouraging high rate of failure for clinical trials in AD has been registered. Researchers raised the critical point of the wrong target and the wrong time to justify these disappointing results. Targeting the wrong pathological alteration means that amyloid-β deposition may be only part of the mechanisms leading to neurodegeneration and that it is not entirely clear which protein conformation (monomeric, oligomeric, fibrillar, amyloid-β plaques) should be attacked. Targeting the wrong time refers to the selected population, including individuals who already have manifested mild or minimal cognitive decline, whose neurodegenerative cascade is ongoing and already unstoppable (Mehta *et al*, 2017). For the latter reason, the last two decades of research focused on identifying individuals at risk for developing AD to provide potential candidates not yet affected by the neurodegenerative processes (Sperling *et al*, 2013).

The concept of MCI became widely accepted after the publication of the Mayo Clinic criteria in 1999. However, it was already identified as a transitional stage between normal aging and dementia in the late eighties, when stage 3 of the Global Deterioration Scale was used to describe subjects in the "predementia stage" (Reisberg *et al*, 1988). In the

original criteria of the Mayo Clinic, an individual with MCI complained about a subjective memory decline, testified by an objective impaired performance at the neuropsychological evaluation, without impact on daily functioning or configuring a dementia condition (Petersen *et al*, 1999). The subsequent revision of the criteria extended the MCI classification to people experimenting with a decline in cognitive domains other than memory, considering that MCI could be underlined by other etiologies than AD (Petersen, 2004). The proposed clinical classification of MCI aided in speculating the possible etiology and partially in predicting prognosis. The amnestic MCI, the most frequent type, traditionally indicated the prodromal stage of typical AD dementia, while the non-amnestic MCI possibly indicated other etiologies such as frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB) (Petersen, 2016). This classification is excessively schematic: the onset of AD dementia can include cognitive deficit other than memory, such as language and frontal disturbances; amnestic MCI subjects do not necessarily progress to AD dementia, but they can convert to other types of dementia; most MCI subjects will never convert to dementia, and some of them will revert to normal cognition (Petersen, 2011). Biomarkers era aided the diagnostic and prognostic definition of MCI, and individuals with cognitive impairment not fulfilling the dementia criteria, but showing biomarkers abnormalities showed a high likelihood of developing AD dementia (Albert *et al*, 2011). Prognostic considerations are essential in evaluating MCI. MCI represents a frequent condition in the elderly, with an estimated overall prevalence of 12-18% in people aged more than 60 years and an estimated progression rate of 5% per year (Petersen, 2016). Several predictors of progression have been suggested and include biomarkers. The Alzheimer's Disease Neuroimaging Initiative (ADNI) is a longitudinal multicenter study aiming to develop clinical, imaging, biochemical, and genetic biomarkers for the early diagnosis of AD launched in 2004 (for up-to-date information, see www.adni-info.org). In the ADNI project, hundreds of individuals with MCI have been followed up for several years to track disease progression. Several studies employing the ADNI databases demonstrated that biomarkers positivity, and including abnormal brain amyloid and tau deposition, hippocampal atrophy, and temporoparietal hypometabolism was associated in MCI to progression to dementia (Prestia *et al*, 2013; Weiner *et al*, 2015; Varatharajah *et al*, 2019; Teng *et al*, 2020). Over decades the concept of MCI has shown to be helpful both in

clinical and research settings, but it is not exempt from criticisms. Controversies continue on the boundaries used to distinguish MCI from normal aging changes in cognitive function. There is enormous variability among studies in neuropsychological evaluation, cutoff scores, and availability of normative data to compare cognitive performances, influenced by age, educational status, and cultural tracts (Díaz-Mardomingo *et al*, 2017). In addition, psychometric evaluation should consider psychosocial factors, such as social isolation and loneliness, personality tract, and factors related to cognitive reserve (Sundström *et al*, 2020). The NIA-AA criteria for MCI proposed that scores on neuropsychological tests should be 1 to 1.5 standard deviation below the mean for the corresponding age and education matched controls, but without providing precise ranges and cutoff scores (Albert *et al*, 2011). Caution in definitions is needed due to the inter-subjects' variability in baseline cognitive performances, which should represent the reference for detecting a potential cognitive decline compared to the previous cognitive and neuropsychological status.

In the last years, growing attention has been focused on the preclinical phase of dementia to improve early AD diagnosis and detect corresponding alterations as soon as possible. The preclinical dementia phase includes subjects between normal aging and MCI, characterized by subtle or absent objective cognitive decline but at risk for developing dementia (Dubois *et al*, 2016). The term Subjective Cognitive Decline (SCD) has been recently adopted to classify individuals previously labeled with a different lexicon, including Subjective Memory Complaints, Subjective Memory Impairment, Subjective Cognitive Impairment (Jessen *et al*, 2020). SCD defines a self-experienced decline in cognitive functions with performance within the normal range on standardized cognitive assessment (Jessen *et al*, 2014). The term "subjective" refers to the self-experienced perception of a cognitive decline, regardless of the cognitive performance. "Cognitive" is extended to all cognitive domains and not limited to memory. "Decline" stands for a subjective impairment concerning a previous cognitive baseline status (Jessen *et al*, 2014). A self-experienced cognitive decline in one or more domains is frequent in older adults, ranging from 25 to 50% in people older than 65 years (van Harten *et al*, 2018; Hao *et al*, 2017; Sánchez-Benavides *et al*, 2018). SCD is most frequently a subjective memory complaint, and people suffering from depression or anxiety tend to over concern about their memory abilities (Si *et al*, 2020; Hill *et al*, 2016). Consequently,

SCD individuals are more likely to manifest anxiety, depression, and neuroticism than subjects without complaints, resulting in poor quality of life (Jenkins *et al*, 2019). It can be challenging to make a precise differential diagnosis between pure SCD and people with concomitant neuropsychiatric and mood disorders concerning their memory. Therefore, emotional and behavioral aspects should always be considered in studies evaluating diagnostic and prognostic repercussions of the SCD condition. Further proof of this association is the co-occurrence of SCD with anxiety and depression associated with more severe present or longitudinal cognitive impact and with a higher risk of developing dementia (Pietrzak *et al*, 2015; Sabatini *et al*, 2021). In SCD, the risk of future cognitive decline increases when the subjective decline is related to memory, the onset of subjective disturbances date less than five years, the subjects is highly concerned about the subjective decline and search for medical help (Jessen *et al*, 2020). However, only 14% of SCD progress to dementia over a long follow-up, and less than one-third progress to the MCI condition (Mitchell & Shiri-Feshki, 2009). The SCD condition may be related to several etiologies, including AD and other neurodegenerative disorders, and the underlying pathological process influences progression (Si *et al*, 2020; Jessen *et al*, 2020). The biomarkers-profile assessment improves the etiological classification of SCD aiding the prediction of outcome. There is evidence that cognitive impairment in SCD is linked to low amyloid-β and high tau levels in CSF (van Harten *et al*, 2013). Brain atrophy in medial temporal and frontal regions is analogously associated with cognitive decline in SCD and predict progression to AD (Perrotin *et al*, 2015). In a similar vein, brain glucose metabolism defects in temporoparietal regions can be detected already in SCD and can predict clinical progression (Scheef *et al*, 2012). In summary, biomarker abnormalities increase the risk of progression to AD dementia (Jessen *et al*, 2014). Therefore, the ATN system applied to the SCD condition can be helpful to predict the future outcome. A recent longitudinal study investigating multiple biomarkers in a large sample of SCD, showed that most participants (56%) had normal biomarkers, while 18% fell within the AD continuum, showing abnormalities in both amyloid-β and tau. Among participants, SCD with amyloid-β positivity had a higher risk of progressing to dementia than participants with normal biomarkers, also showing a dose-response pattern related to the number of biomarkers affected (Ebenau *et al*, 2020).

Besides SCD, the term pre-MCI has been used to identify individuals with subtle cognitive decline, representing a decline from a previous level of function, not fulfilling the criteria for MCI (Storandt *et al*, 2006). The categorization of pre-MCI individuals highlights the discrepancy between a clinical judgment of MCI and neuropsychological performances within normal ranges (Duara *et al*, 2011). An operational definition was adopted to include participants in clinical studies, comprising individuals with a Clinical Dementia Rating scale (CDR) score of 0.5 and neuropsychological performances above -1.5 according to age and education norms, revealing subtle cognitive deficits (Duara *et al*, 2011). The pre-MCI classification does not include etiological considerations, and the prognostic importance of this construct still needs confirmations since pre-MCI may indifferently progress to AD, to other dementia, or not progress at all (Loewenstein *et al*, 2009). At 2-3 years, cognitive impairment progression has been reported for less than 30% of subjects, confirming the need for a multimodal approach to identify the potential risk/protective factors for cognitive decline in this population (Duara *et al*, 2011). The presence of neuropsychiatric symptoms, including behavioral and psychiatric disturbances, seems to be associated with the progression of cognitive decline (Ismail *et al*, 2016). Although extensive utilization of biomarkers in SCD and cognitively unimpaired elderly, several critical points should be addressed to correctly understanding data on the preclinical dementia stage. There is a need for a standard terminology and assessment, as proposed by the recent consensus on SCD (Jessen *et al*, 2020). Since SCD and pre-MCI represent heterogeneous populations, only a comprehensive assessment including neuroimaging, CSF measures, clinical and neuropsychological characterizations may improve accuracy in defining the clinical outcome (Si *et al*, 2020). The assessment of biomarkers of neurodegeneration and pathology in the SCD/pre-MCI categories combined with extended follow-up can be expected to provide helpful information about the risk profile by identifying subjects who are not on a trajectory to dementia, avoiding exposure to possible side effects of the tested treatment.

Biomarkers in Alzheimer's disease and other neurodegenerative disorders

Pathological protein deposition of amyloid-β in the senile plaques and tau protein in the neurofibrillary tangles are crucial in AD pathophysiology. The detection of these

pathological alterations, especially at the early stage of disease, contributes to diagnostic workup and prognostic stratification (Blennow & Zetterberg, 2018). Besides biomarkers of neuropathology, markers of neurodegenerative changes have been proposed to support AD diagnosis (Dubois *et al*, 2014). Biomarkers in the clinical setting are needed to improve the diagnostic workup of several neurodegenerative conditions. A timely diagnosis of AD and other neurodegenerative disorders is needed for prognostic consideration and to optimize patient management, with misdiagnosis causing poor treatments and resource consumption. Especially in the AD continuum, the early identification of individuals at risk of developing dementia allows the inclusion of subjects in clinical trials (Hansson, 2021). Studies in autosomal dominant AD forms showed that amyloid-β starts to accumulate decades before the onset of symptoms (Bateman *et al*, 2011). Tau deposition in neurofibrillary tangles, confined to the medial temporal lobe, is a common finding in individuals over 60 years, but in AD, it spreads to the cortical regions, possibly driven by amyloid-β pathology (Peng *et al*, 2020). Therefore, disease-modifying therapies targeting amyloid-β or tau are currently under evaluation, but results have been disappointing. Multiple biomarkers should be considered to avoid mistakes in subject selection for clinical trials, allowing for recognizing co-pathology and mixed pathology. In addition, the included biomarkers should have enough sensitivity and specificity in the prodromal or pre-symptomatic phase to detect alteration before irreversible neurodegeneration. Lastly, surrogate biomarkers should be developed to monitor response to therapy (Hansson, 2021). Currently, biomarkers of pathology in AD include measurement of amyloid-β and tau protein, while the most recognizable downstream biomarkers are represented by cortical atrophy, hypometabolism, and CSF neurofilaments.

Amyloid-β pathology can be detected by amyloid-PET or by CSF measurements. Amyloid-PET allows the visualization of insoluble amyloid-β fibrils aggregated in the senile plaques (Sabri *et al*, 2015; Clark *et al*, 2012). Amyloid-β plaques start to accumulate in neocortical association areas, preferentially in medial parietal and frontal regions (Gordon *et al*, 2018) (see **Figure 3**). The exclusionary role of amyloid-PET is unique, and a negative result excludes AD (Marcus *et al*, 2014). However, there is consistent evidence that amyloid-PET positivity can also be detected in cognitively normal elderly. Markedly positive cases are characterized by high amyloid-PET tracer

binding in AD signature regions, such as the posterior cingulate cortex and the precuneus, but without producing a noticeable impact on cognitive functions. Overall, the rate of amyloid-PET positivity in normal adults ranges from 10 to 30% (Chételat *et al*, 2013). In addition, there is a considerable percentage of cognitively normal adults with borderline values of brain amyloidosis, whose scan positivity depends on the quantification method and the selected cutoff (Suppiah *et al*, 2019). The population with intermediate cortical amyloid load might represent another considerable percentage of cognitively normal elderly, estimated at 25%, further increasing uncertainty regarding the use of amyloid-PET as the only screening tool for clinical trials (Chételat *et al*, 2013).

In CSF, several amyloid-β configurations can be measured, including the amyloid-β40 and amyloid-β42 peptides. The ratio amyloid-β42/amyloid-β40 showed high concordance with amyloid-PET and predicted with high accuracy progression to AD in the MCI population (Mattsson *et al*, 2017). The levels of amyloid-β42 decrease significantly in AD, even before detecting cortical amyloid deposition using amyloid-PET (Palmqvist *et al*, 2016). The main limitation in using CSF measurements is the collection of samples by lumbar puncture, which is an invasive procedure not widely available. The validation of the plasma dosage of amyloid-β42 and amyloid-β40 is ongoing, but plasma levels are less sensitive than CSF levels and more dependent on the selected cutoff (Schindler *et al*, 2019).

Tau pathology can be *in vivo* delineated with both PET and CSF analysis, similarly to amyloid-β. The accuracy of tau-PET in discriminating AD patients from controls resulted higher than MRI and its specificity higher than amyloid-PET and CSF measurement of amyloid-β42/amyloid-β40 (Ossenkoppele *et al*, 2018) (see **Figure 3**). Tau deposition correlates with cognitive impairment and neurodegeneration. Thus tau-PET imaging can differentiate AD phenotypes, showing temporoparietal tau pathology in the typical amnestic AD, posterior parietal and occipital tau deposition in the PCA, an asymmetric left temporal high signal in the logopenic variant PPA, widespread uptake sparing temporoparietal regions in the frontal variant of AD (Vogel *et al*, 2021). In CSF, the most widely studied tau species are the t-tau and p-tau. P-tau is elevated in AD and not in other neurodegenerative disorders, while t-tau represents an unspecific marker of neurodegeneration (Blennow & Zetterberg, 2018). P-tau can also be measured in plasma, showing high accuracy in AD diagnosis (Palmqvist *et al*, 2020) and predicting AD

progression in MCI samples (Janelidze *et al*, 2020), and also being correlated with the cortical deposition of senile plaques and neurofibrillary tangles (Mattsson-Carlgren *et al*, 2021). However, due to the lack of unquestionability, plasma markers of pathology are not currently recommended in clinical practice (Dubois *et al*, 2021).

Markers of neurodegeneration include MRI, [^{18}F]FDG-PET, and fluid biomarkers. Structural MRI is widely used to assess patterns of brain atrophy and to visualize regional neuronal loss. The earliest neurodegeneration signs in AD can be detected in the medial temporal lobe, the entorhinal cortex, the hippocampus, and para-hippocampal structures (Lombardi *et al*, 2020) (**Figure 3**). Neuronal damage in the hippocampus manifests as a reduction of hippocampal volume. Therefore, several methods have been developed to provide a volumetric measure of this region in AD (van Oostveen & de Lange, 2021). Reduction in hippocampal volume represents an early marker of AD pathology, even if not specific for AD (Vijayakumar & Vijayakumar, 2013). In addition, also normal brain aging is associated with reduced brain volume, not affecting cognitive functions (Fjell *et al*, 2014). This aspect imposes caution when using normality volume thresholds. Hippocampal volume reduction starts years before symptoms onset, and the detection of abnormal atrophy in this region is a sensitive marker for AD in cognitively normal elderly (Apostolova *et al*, 2010). The segmentation of the hippocampus may be obtained with manual or automatic methods. Automatic segmentation allows defining normative data and cutoff for normality and pathology and favors the reproducibility of results (De Francesco *et al*, 2021). FreeSurfer is a software providing accurate and reliable quantification of the hippocampal volume (Fischl, 2012). After normalizing the intracranial volume, single-subjects specific hippocampal volume can be compared to the reference volume of a population of individuals with normal cognition. This method allows identifying individuals below the fifth percentile of the normal hippocampal volume population, thus considered to have abnormal hippocampal volume and at risk for cognitive impairment (De Francesco *et al*, 2021).

[^{18}F]FDG-PET is suitably used in clinical practice due to its potential in revealing specific patterns of neurodegenerative changes, underlined by hypometabolism brain regions, in different neurodegenerative conditions. However, the diagnostic accuracy of [^{18}F]FDG-PET depends on the employed methods to quantify brain metabolism signals (Chételat *et al*, 2020). In AD, it has been consistently reported that brain hypometabolism

correlated with brain atrophy and is associated with tau deposition, reflecting neurodegenerative changes (**Figure 3**).

Several fluid biomarkers resulted useful to reveal neurodegeneration *in vivo*, including t-tau and neurofilaments, which are detectable both in CSF and in blood and are elevated in case of axonal degeneration, especially in AD, Parkinson's Disease (PD), FTD, and amyotrophic lateral sclerosis (ALS) (Oosterveld *et al*, 2020; Ashton *et al*, 2021). CSF levels of neurofilaments can be normalized by suitable disease-modifying treatment in multiple sclerosis, thus representing a potential marker to monitor response to therapy (Delcoigne *et al*, 2020).

Biomarkers for neurodegenerative conditions other than AD showed promising results but still need further confirmation. Neurofilaments in ALS have been used to reveal neurodegenerative changes, and their levels are constantly elevated in CSF of patients with motor neuron disease, but still, they cannot be used for diagnostic purposes (Li *et al*, 2016). In the α-synuclein spectrum, the measurement of α-synuclein in fluid biomarkers may have diagnostic potential. In CSF, α-synuclein levels are decreased overall in PD, DLB and multiple system atrophy (MSA), but with insufficient accuracy in discriminating patients from healthy controls or other neurodegenerative disorders (Parnetti *et al*, 2019). Research on more precise α-synuclein detection essays is ongoing, also aiming to differentiate between different parkinsonian syndromes linked to different α-synuclein conformations (Singer *et al*, 2020). However, the lack of PET tracer to detect cortical diffusion of Lewy bodies in living patients is still a limitation. Currently, there are no reliable biomarkers for non-AD tauopathies or other brain misfolded protein aggregates. The development of blood-based markers is hugely encouraging since they could potentially dramatically reduce costs for screening candidates in clinical trials and enlarge the studied population. The high expenditure of time and resources is the main limitation in the simultaneous use of multiple methods to characterize neurodegenerative diseases, which can be delineated with spectacular accuracy by multimodal approaches, as shown in **Figure 3**. Multimodal technique studies highlighted that the combination of several biomarkers may improve diagnostic and prognostic accuracy, and the correspondence between different markers of pathology and neurodegeneration help in revealing mechanisms leading to neurodegeneration. Implementing multiple, reliable, cost-effective, and accessible biomarkers needs further improvements, specific

standardization of tests and cutoff, the development of standard operating procedures, the elaboration of appropriate-use criteria, validation in longitudinal studies (Hansson, 2021). All these steps are essential for the development and monitoring of disease-modifying therapies.

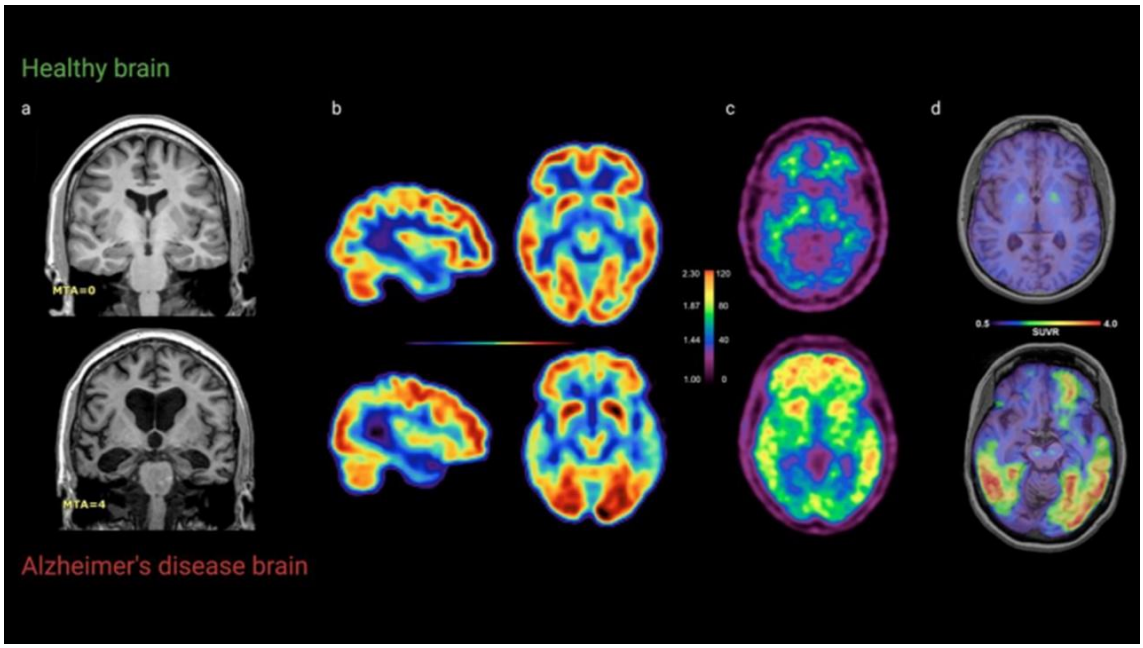


Figure 3. Neuroimaging of the healthy brain versus Alzheimer's disease, with structural MRI, [^{18}F]FDG-PET, amyloid-PET, tau-PET (source: van Oostveen et al., 2021).

Controversies about the biological definition of Alzheimer's disease

The NIA-AA framework represented a fundamental shift in AD definition and diagnosis. However, the pure biological definition of AD has several criticisms. The most recent recommendations of the IWG on the clinical diagnosis of AD discuss these critical issues and define AD as an indivisible clinical and biological diagnosis, in which the specific clinical phenotype accompanies the biological evidence of AD pathology (Dubois *et al*, 2021). There are several reasons for considering crucial the integration between clinical picture and biomarker alterations. The progressive aging of the world population provoked an increased burden due to AD and other neurodegenerative dementia (Brayne & Miller, 2017). The availability of *in vivo* biomarkers increased the diagnostic accuracy of these neurodegenerative conditions. There is now considerable evidence that AD can be associated with other neuropathology conditions, such as α-

synuclein deposition, vascular alterations, non-AD tau pathology (especially argyrophilic grain disease), TAR DNA-binding protein-43 (TDP-43) accumulation (Robinson *et al*, 2018; Karanth *et al*, 2020). Co-pathology in AD dementia is particularly frequent, considering that pure AD pathology as revealed by autoptic examination is responsible for about one-third of clinically suspected AD (Robinson *et al*, 2018). In individuals with high cortical amyloid load, mixed pathology or other pathological proteins deposition can be the main responsible for the clinical symptoms (Karanth *et al*, 2020; Schoemaker *et al*, 2021). On the other hand, amyloid-β pathology can be associated with other neurodegenerative conditions, including PPA, DLB and FTD (Kantarci *et al*, 2017; Bergeron *et al*, 2018; Perry *et al*, 2017). Besides pathological overlap, blurred clinical presentations may be possible. The amnestic phenotype is not exclusive of AD, and it can be the primary clinical disturbance in other neuropathological entities, including DLB, cerebral amyloid angiopathy (CAA), the limbic-predominant age-related TDP-43 encephalopathy (LATE), and vascular dementia (Schoemaker *et al*, 2021; Nelson *et al*, 2019; Moylett *et al*, 2019). In particular, the recently characterized LATE has been proposed to be the primary etiology of memory impairment in older individuals with or without concomitant amyloidopathy (Nelson *et al*, 2019). In addition, isolated amnesia may be the unusual presentation of several tauopathies, such as FTD, corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and primary age-related tauopathy (PART) (Bell *et al*, 2019; Sakae *et al*, 2020; Hornberger & Piguet, 2012; Brown *et al*, 2010). Hence, in all these conditions, amyloid-β biomarker positivity is not necessarily a synonym of AD. Coherently, uncommon phenotypes of AD should not be classified as having AD, even when showing the biomarkers evidence of amyloid-β and tau pathology, considering that other pathology substrates may be responsible for the clinical picture. The lack of a sharp and unquestionable biological classification becomes critical when defining the preclinical stage of AD due to the possibility of novel therapeutic strategies targeting AD pathology before the onset of symptoms. Even when presenting both amyloid-β and tau pathological alteration, individuals without cognitive impairment are not invariably expected to develop AD dementia (Jack *et al*, 2019; Hanseeuw *et al*, 2019). This is particularly true with advancing age, which is associated with an increased rate of amyloid-β pathology (Braak *et al*, 2011). Most elderly showing AD pathological changes will never develop dementia due to favorable lifestyle, genetic,

and other brain factors preventing cognitive decline (Arenaza-Urquijo & Vemuri, 2018). In all these conditions, amyloidopathy is not sufficient to define AD without clinical correspondence (Dubois *et al*, 2021).

Other practical issues impose limitations to the solely biomarker-based definition of AD. There are several methodological caveats, and the principal regards thresholding normality and pathological level of markers. In most cases, the definition of a binary threshold is not accurate enough since both amyloid-β and tau deposition represent a continuum rather than a dichotomic variable. This assumption is testified by the presence of a small amount of amyloid-β pathology in middle-aged people without cognitive impairment that can have subtle or absent brain structural and functional changes (Milà-Alomà *et al*, 2021). In addition, laboratory differences in CSF analysis or the employment of different PET tracers impede the definition of a universally validated cutoff for measures of amyloid-β and tau pathology. The problem to identify a clear threshold regards not only the biological but also the clinical definitions. Normality thresholds in psychometric evaluation can be lowered to detect early or subtle deficits. However, this approach implies a loss of specificity (Dubois *et al*, 2021). Lastly, the use of CSF analysis or PET scan for clinical purposes implies high costs, and it is not universally accessible worldwide (Ritchie *et al*, 2017). Some ethical considerations need to be addressed concerning biomarkers positivity in people with normal cognition. The search for markers of AD pathology in cognitively unimpaired individuals is not recommended in clinical practice due to the unsure clinical progression to dementia, the absence of modifiable risk factors, and the lack of curative therapies (Guerra *et al*, 2015).

Other major forms of neurodegenerative dementia

Neurodegenerative diseases are a wide range of brain disorders characterized by progressive neuronal loss triggered by different anatomopathological alterations. The recognizable pathobiological mechanism can lead to a specific neurodegenerative disorder. However, both *post-mortem* and *in vivo* studies employing biomarkers showed that the same neuropathological mechanism could trigger different clinical phenotypes. On the other hand, similar clinical syndromes can be due to different underlying mechanisms of neuropathological alterations. This evidence promoted a conceptual

spectrum-based approach, where neurodegenerative diseases, classified as proteinopathies, belong to a spectrum characterized by the deposition of specific pathological protein aggregates (Perani *et al*, 2019a; Elahi & Miller, 2017). For neurodegenerative dementia, this approach identifies six main groups of proteinopathies, including the amyloidopathy, microtubule-associated protein tau (MAPT) pathology, TDP-43, fused in sarcoma (FUS) protein, α-synuclein, and prion protein (Elahi & Miller, 2017). Furthermore, the presence of pathological protein deposition usually anticipates the clinical onset of symptoms by years, theoretically providing the possibility to identify pathological alterations indicative for neurodegenerative dementia in a preclinical phase. **Figure 4** represent a clinic-pathological classification of proteinopathies.

AD, the most frequent neurodegenerative dementia, is pathologically characterized by the extracellular deposition of amyloid-β plaques and the intracellular accumulation of tau protein neurofibrillary tangles (Braak *et al*, 2011). The typical AD manifests with insidious onset of memory deficits, with progressive involvement of other cognitive domains, including language, visuospatial and executive functions (McKhann *et al*, 2011). In addition, atypical presentations can be possibly related to AD pathology, which are the PCA, dominated by visuospatial and perceptual disturbances, the logopenic variant PPA, characterized by impaired naming, word-finding disruption, and working memory deficit, and the frontal AD variant, mainly characterized by executive and behavioral disturbances (Dubois *et al*, 2014). The various clinical syndromes are associated with a specific topographical distribution of the neurodegenerative changes (see **Figure 5**). Typical AD is associated with neurodegeneration in medial temporal lobe and temporo-parietal regions. The logopenic variant PPA presents neuronal loss in the posterior perisylvian area, usually in the left hemisphere. PCA is characterized by neurodegeneration in the occipital and posterior parietal regions. The frontal AD variant shows degenerative changes in temporo-parietal and frontal regions. Furthermore, based on the age of onset of cognitive deficit, AD patients can be classified in late-onset AD (LOAD), the typical amnestic phenotype classically starting later than 65 years, and early-onset AD (EOAD), when the onset occurs before 65 years. The latter phenotype is usually characterized by faster disease progression, frequent atypical onset, and more severe neuropathology than the LOAD phenotype (Mendez, 2017).

Frontotemporal lobe degeneration (FTLD) is an umbrella term including several pathological alterations causing neurodegeneration within the frontal and temporal lobes of the brain. The term comprises FTD, the atypical parkinsonism PSP and CBD, and the complex FTD associated with Motor Neuron Disease (FTD-MND) (Lashley *et al*, 2015). Tau and TDP-43 aggregates cause most FTLD cases. Intracellular FUS deposits can be found in fewer cases (Mackenzie *et al*, 2010). Even if each pathological substrate is generally associated with a specific disorder, overlap may exist. Therefore, tauopathies can be classified according to the specific tau conformation. Six isoforms of tau are present in the human brain, consisting of three repeats (3R) or four repeats (4R) of the microtubules-binding domain. In AD, there is an equal ratio of 3R:4R tau. In PSP, CBD, and argyrophilic grain disease, the most predominant pathology is the 4R tauopathy. FTD presents variable pathology, and Pick's disease is a 3R predominant tauopathy (Irwin, 2016). Genetics plays a fundamental role in the development of FTLD. One-third of patients with FTD have a family history of dementia. The most frequent mutations associated with the development of FTLD include C9orf72, progranulin, TDP-43 mutations, and MAPT mutations which provoke tau pathology. FTD–MND is the most heritable subtype (Ghasemi & Brown, 2018). Clinically, there are three principal FTD syndromes, characterized by progressive development of executive and behavioral disturbances and impaired motor and language abilities, representing the most frequent neurodegenerative dementia in people under 65 years (Bang *et al*, 2015). Specifically, the behavioral variant of FTD (bvFTD) is the most frequent clinical syndrome, characterized by behavioral and mood disturbances associated with deficits in executive functions; two language syndromes can be recognized, the non-fluent/agrammatic variant of the PPA, characterized by non-fluent speech, agrammatism, and phonemic errors, and semantic dementia (SD), dominated by deficits in word recognizing and understanding and often associated with behavioral abnormalities (Neary *et al*, 1998). Neuropathologically, bvFTD can be highly heterogeneous, including tau, TDP-43, and FUS aggregates, while SD is strictly associated with TDP-43 (type C) inclusions (Mann & Snowden, 2017). When motor disturbances are the main sign, PSP, CBD or FTD-MND should be suspected. FTD-MND represent about 15% of FTD cases, clinically characterized by behavioral and executive disturbance associated with ALS manifestations, which include muscle weakness, fasciculations, spastic tone, hyperreflexia, dysarthria, dysphagia, and

respiratory failure, due to the degeneration of motor neurons in the brain and the spinal cord (Olney *et al*, 2017). PSP is atypical parkinsonism characterized by a rigid-akinetic syndrome with impaired balance, early falls, vertical supranuclear gaze palsy, and bulbar disturbances, such as dysarthria and dysphagia. PSP patients often manifest behavioral, neuropsychiatric, and cognitive disorders, such as emotional lability, anxiety, depression, apathy, impulsivity, and executive dysfunction (Rowe *et al*, 2021). CBD is pathologically defined as a tauopathy, whose most common presentation is the corticobasal syndrome (CBS). CBS is characterized by a rigid-akinetic syndrome associated with limb apraxia, dystonia, and cognitive signs, frequent acalculia, visuospatial deficits, and alien limb phenomenon (Armstrong *et al*, 2013). The clinical picture configuring CBS can also be caused by AD pathology. Thus, it is not trivial to differentiate between the pathological disorder, CBD, and CBS's clinical disorder. Other common manifestations of CBD include the non-fluent variant PPA, a behavioral syndrome, and PSP (Constantinides *et al*, 2019).

The α-synuclein spectrum includes PD, PD dementia, DLB, and MSA, sharing the same neuropathology, namely deposition of α-synuclein in the intracellular aggregates called Lewy bodies (Brás *et al*, 2020). The deposition of α-synuclein follows a precise scheme in each synucleinopathy, affecting the dopaminergic pathways in PD, widely involving cortical areas in PD dementia and DLB, hitting the oligodendrocytes in cortical and subcortical regions in MSA (McCann *et al*, 2014). All α-synucleinopathies are characterized by common symptoms, including motor disturbances related to extrapyramidal dysfunction and several non-motor signs associated with autonomic impairment and cognitive and neuropsychiatric disturbances (Brás *et al*, 2020). PD has been considered a pure motor disorder for many years, not associated with cognitive decline. However, recent and substantial evidence suggests that subtle or minimal cognitive impairment can be present at the onset of PD in about 20% of patients, and about 80% of patients will develop cognitive dysfunction over the disease course (Aarsland *et al*, 2009; Dubbelink *et al*, 2014). Cognitive decline in PD can be heterogeneous and primarily involves attention and executive functions, but it can be associated with impairment in memory and visuospatial abilities (Fang *et al*, 2020). PD dementia is a late manifestation of PD affecting up to 80% of PD patients after 20 years of disease. From a clinical perspective, PD dementia and DLB are challenging to

distinguish. Historically, the main difference between the two disorders has been the onset of cognitive disturbances, early (within one year) for the DLB and late for the PD dementia, but exceptions may exist (Jellinger & Korczyn, 2018). In addition, DLB core features, which include cognitive fluctuations, visual hallucinations, and parkinsonism, may be present also in PD dementia (Jellinger & Korczyn, 2018). Lastly, in PD dementia, neuropsychiatric disturbances are more common than in DLB and include apathy, depression, and anxiety appear more commonly than in DLB patients, while irritability, behavioral disturbances, and neuroleptic hypersensitivity are frequent in DLB (Fang *et al*, 2020). MSA is characterized by prominent autonomic disturbances associated with akinetic-rigid parkinsonism or cerebellar ataxia, respectively, due to basal ganglia or cerebellar involvement. While dementia has been considered a non-supporting feature in MSA for many years, consistent evidence showed a wide range of cognitive symptoms during the disease course, including executive dysfunctions as the most common presentation, often associated with memory and visuospatial disturbances (Stankovic *et al*, 2014).

Prion diseases are a rare group of neurodegenerative conditions associated with rapidly progressive and invariably fatal dementia. Neuronal loss is determined by the toxic effect of the brain prion protein (PrP^{C}, where "C" stands for cellular protein), which assumes an abnormal misfolded conformation (PrP^{Sc} where "Sc" stands for scrapie, the prion disease of sheep and goats) (Colby & Prusiner, 2011). Prion dementias can be sporadic, genetic, or acquired diseases. About 90% of cases are the sporadic Creutzfeldt-Jakob disease, about 10% are genetic and include the familial Creutzfeldt-Jakob disease, the Gerstmann-Straussler-Scheinker syndrome, and the Fatal Familial Insomnia, and less than 1% are acquired cases and include the variant of the Creutzfeldt-Jakob disease, iatrogenic Creutzfeldt-Jakob disease, the Kuru (Geschwind, 2015). Among these disorders, the most common is the sporadic Creutzfeldt-Jakob disease, which is clinically characterized by the subacute onset of rapidly progressive dementia associated with myoclonus, visual or cerebellar disturbances, pyramidal or extrapyramidal signs, and akinetic mutism. Cognitive deficits include memory disturbances, aphasia, executive and visuospatial deficits, apraxia, decreased alertness (Tee *et al*, 2018). The detection of brain PrPSc allows definite diagnosis; however, co-pathology may also exist in prions disease, where amyloid-β deposition, tau pathology, and microglia activation have been reported in

preclinical, *post-mortem* and *in vivo* studies (Iaccarino *et al*, 2018b, 2018a; Rossi *et al*, 2019; Day *et al*, 2018).

The classification of neurodegenerative dementia in proteinopathies aims to facilitate the diagnostic framework and reveal common neurodegeneration mechanisms (see **Figure 4**). However, patients with different neurodegenerative dementias may present similar clinical phenotypes, making the diagnostic workup challenging. In this vein, the biomarker era opened a new possibility in defining pathological etiologies of neurodegenerative disorders. Thus, tools able to underline, at the same time, specific protein deposition and other molecular and biological processes, such as PET, represent crucial instruments in supporting *in vivo* diagnosis of several cognitive disorders.

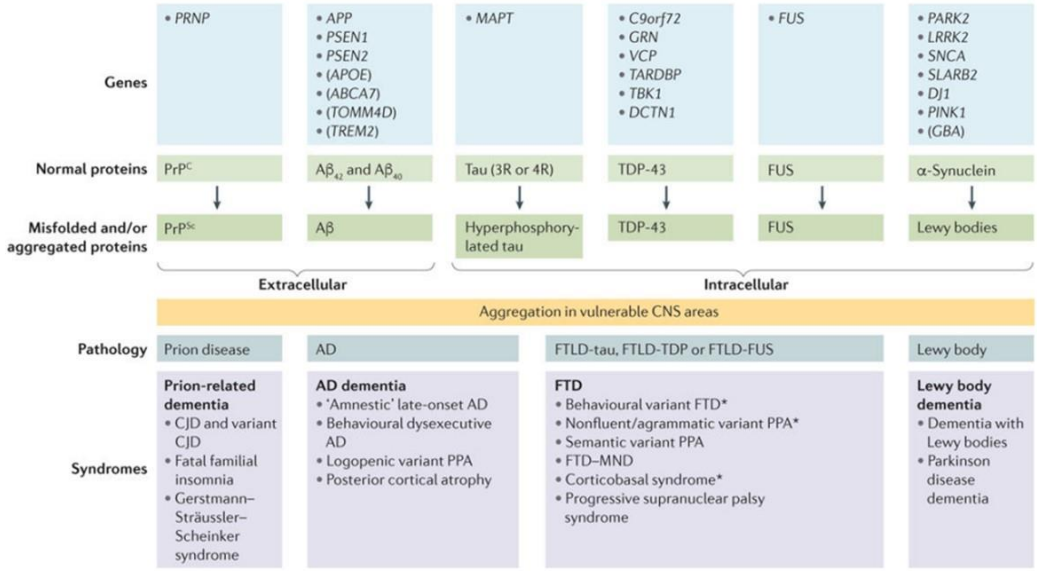


Figure 4. Clinical-pathological classification of neurodegenerative disease characterized by abnormal deposition of misfolded proteins (source: Elahi et al., 2017).

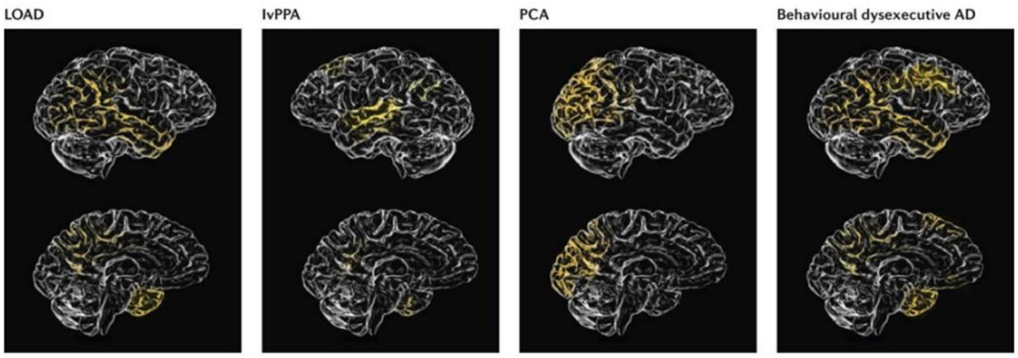


Figure 5. Patterns of neurodegenerative changes associated with different Alzheimer's disease phenotypes. Temporo-parietal regions are mainly involved in the typical late-onset AD. The logopenic PPA is characterized by neuronal loss involving the parietal lobe and the perisylvian region. The PCA shows neurodegenerative changes involving the posterior parietal and occipital regions. The frontal AD variant is characterized by coupled neuronal dysfunction in the AD signature regions, namely temporo-parietal cortices, and in frontal lobes (source: Elahi et al., 2017)**.**

Neuroinflammation and Neurodegeneration

Neurodegenerative diseases represent a wide range of brain disorders characterized by progressive, inexorable neuronal loss and neuronal death starting in specific regions of the CNS. The neuronal death represents an irreversible event in the neurodegenerative process since neurons are unable to regenerate. Despite remarkable progress in understanding the pathogenic mechanisms underlying neurodegeneration, a complete and clear picture is far to achieve. Several environmental factors, lifestyle, and genetic, may be involved. At the pathological level, neurodegenerative diseases are characterized by abnormal protein deposition, exaggerated oxidative stress, mitochondrial dysfunction, and dysregulation in neuroinflammatory processes (Chitnis & Weiner, 2017). Neuroinflammation can be primarily considered a protective mechanism, aiming at repairing damaged tissue, eliminating pathogens, removing abnormal protein deposits and cellular debris (Comi & Tondo, 2017). Neuroinflammatory responses involve several immune and inflammatory cells, including microglia, astrocytes, lymphocytes, mast cells, and inflammatory molecules, such as cytokines, chemokines, and growth factors (Guzman-Martinez *et al*, 2019). While initially protective, chronic and sustained activation of neuroinflammatory cells provokes detrimental consequences, which reflect on neuronal dysfunction, synaptic degeneration, and neuronal death, the basis of neurodegeneration. Inflammatory responses in CNS become persistent and deleterious depending on endogenous or environmental factors, such as genetic mutations, aging, brain disorders including trauma, stroke or infections (Stephenson *et al*, 2018). It has been suggested that neuroinflammation may drive neurodegeneration through several mechanisms, counting apoptosis caspase-mediated, necroptosis (which is independent of the caspase activation), autophagy, retrograde degeneration, Wallerian degeneration, demyelination, and astrogliopathy (Chitnis & Weiner, 2017). Regardless of the pathogenic mechanisms, neuroinflammation has been associated with promoting neurodegeneration in several neurodegenerative diseases, especially in AD, PD, and ALS. The presence of glial cells in specific regions affected by neurodegenerative changes testifies that neuroinflammation is coupled to neurodegeneration, even if this relationship has not been fully elucidated yet.

The Central Nervous System as an immune-privileged organ

Specific features of the Central Nervous System (CNS), including the presence of the Blood-Brain Barrier (BBB), the absence of a lymphatic system, and peculiar regional immune cells, the microglia, led to the belief that the brain represented an immune-privileged site. However, this concept is only partly true, and evidence from the past decades shows that a complex interplay between central and peripheral innate and adaptative inflammatory responses regulates brain neuroinflammation, which cannot be considered an isolated mechanism (Bettcher *et al*, 2021).

The BBB is a diffusion barrier regulating the transfer of cells and molecules from the blood to the brain. Endothelial cells, astrocytes, and pericytes contribute to form this diffusion barrier, which selectively hampers the passage of most blood-originated substances, participating in brain homeostasis and regulating efflux and influx transfer (Sharma *et al*, 2018). The restrictive access to brain parenchyma by leukocytes and antibodies is finely regulated, and it is not absolute: several types of stimuli can modify the permeability of the BBB, including various inflammatory mediators whose production is increased in brain injury (Mizee & de Vries, 2013). BBB disruption causes influx into the CNS of neurotoxic molecules and inflammatory cells, facilitating neuronal injury, synaptic dysfunction, and neurodegeneration (Sharma *et al*, 2018).

The Glymphatic system is a recently characterized system promoting clearance of waste proteins and metabolites from the CNS. It favors the distribution to the brain of critical metabolic compounds, including glucose, amino acids, lipids, and neurotransmitters (Benveniste *et al*, 2020). A continuous interchange between the CSF and the interstitial fluid is guaranteed throughout this system, facilitating the clearance of interstitial solutes to the perivenous drainage structure. The glymphatic system is of particular interest for research in neurodegenerative disease, and especially in AD. Since its initial description, it was evident that the elimination of β-amyloid would be favored by an efficient glymphatic system (Iliff *et al*, 2012). In addition, a peculiar feature of the glymphatic system is that its functions are carried out essentially during sleep. Thus, sleep serves vital functions, facilitating the elimination of potentially neurotoxic molecules, including β-amyloid (Cordone *et al*, 2019). This is of crucial importance: in a mouse model of AD, sleep was associated with a 60% increase in the interstitial brain space,

reflecting an increased rate of convective exchange from the CSF to the interstitial space and resulting in accelerated clearance of β-amyloid (Xie *et al*, 2013).

Alterations of the BBB and abnormalities in the glymphatic system functions provoke the accumulation of neurotoxic molecules and stimulate an inflammatory response. The production of neurotoxin cytokines activates resident inflammatory cells. In addition, the innate immune response can participate in the reaction to the insults, producing infiltration into the CNS of macrophages, neutrophils, and lymphocytes. Thus, when the immune-privileged condition is lost, the CNS becomes the scene of deleterious neuroinflammatory responses (Stephenson *et al*, 2018).

The innate and adaptive immunity in the Central Nervous System

All cells populating the CNS can contribute to the neuroinflammatory response, either directly or producing factors able to modify the environment in which the same cells act. However, innate immunity, which represents the first line of immune defense, is centered on the activity of the glial cells. Glial cells include astrocytes, oligodendrocytes, and microglia, which perform their activity primarily to support and sustain neurons.

Microglia are the primary resident innate immune cells of the CNS and are ubiquitously distributed in the brain. They play an essential role in homeostasis and host defense by detecting changes in the environment, migrating to damaged sites, phagocyting cellular debris, participating in myelin maintenance, and reacting against pathogenic stimuli (Kwon & Koh, 2020). Thus, microglia are primarily phagocytic cells providing surveillance and scavenging function, but they also take part in synaptic remodeling by engulfing discarded material and mediating synaptic pruning, which is a fundamental process in synaptic maturation (Paolicelli *et al*, 2011). During developmental phases, a deficit in microglia results in abnormalities in synaptic transmission, functional brain connectivity, and consequent neurodevelopmental and neuropsychiatric disorders such as autism, repetitive-behavioral and social interaction disturbances (Ma *et al*, 2014). Microglia can identify micro-environmental changes throughout a specific system, the "sensome", which allows the detection of endogenous ligands and pathogens, and thus modulates the specific immune response (Hickman *et al*, 2013). Several kinds of insult can activate microglia, which respond to the environment

by shifting into pro-inflammatory or anti-inflammatory phenotypes and in turn sustain or switch off neuroinflammatory response, modulating the production of cytokines, interleukins (IL), adhesion, and other inflammatory molecules (Heneka *et al*, 2015). Microglia express receptors can recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These signals are activated by the presence of damage in a tissue or by external pathogens, and they can be actively secreted by cells or released after cell damage and death to activate the immune system (Stephenson *et al*, 2018). The most recognized PAMPs are the Gram-negative lipopolysaccharide (LPS) cell-surface antigen and other bacterial lipopeptides, while DAMPs include endogenous molecules such as nuclear DNA, the adenosine triphosphate, IL-1, and amyloid-β (Venegas & Heneka, 2017). PAMPs and DAMPs are ligands for the pattern recognition receptors, a large receptors family including the scavenger receptors and the Toll-like receptors (TLRs), mainly expressed on the plasma membrane, and the nuclear oligomerization domain-like receptors (NLRs), which are usually expressed in the cytoplasm (Kigerl *et al*, 2014). Microglia cells respond to endogenous and exogenous stimuli and modulate their activity depending on the stimulus, the activated pathway, the disease, and the disease phase. Thus, their activation involves multiple mechanisms and possibly results in multiple phenotypes (Heneka *et al*, 2015). Microglia are usually schematically classified as pro-inflammatory (M1) and anti-inflammatory (M2). M1 cells act in a pro-inflammatory environment characterized by increased levels of tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-12, IL-18, which are predominant in chronic inflammation. IL-4, IL-10, IL-13, and the Transforming Growth Factor (TGF)-β, instead, are predominant in the anti-inflammatory state. In this state the M2 phenotype is mainly expressed, and phagocytic activity of microglia is increased. These two opposite states represent only the polar extremes of microglia activation. Microglia react to different types of neuronal injury throughout the expression of multiple phenotypes, able to modify the microenvironment secreting specific factors (Bachiller *et al*, 2018). Some of these phenotypes play a protective role, maintaining homeostasis and remodeling synapses; some play harmful functions, such as enhancing local chronic neuroinflammation; others are in a quiescent, resting state; others may play multiple detrimental and protective functions (Dubbelaar *et al*, 2018) (see **Figure 6**). In response to the stimulus, different pathways can be activated. LPS exposure can activate the TLRs pathway, which is

associated with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcriptional factor modulating inflammatory response, favoring the expression of pro-inflammatory cytokines such as interferon-γ (IFNγ) IL-1, IL-6, and TNF-a (Kany *et al*, 2019). Pathogens detection by the pattern recognition receptors expressed by microglia may also result in the activation of the Janus Kinase (JAK)-signal and activator of transcription (STAT) pathway, which induce expression of the IFNs, able to favor both cytokines production and contemporary to resolve inflammation by inhibiting TLRs (Ivashkiv & Donlin, 2014). Overall, neuroinflammation can be considered a protective response. Even the M1 activation, leading to the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, which enhance inflammation and attract other immune cells in the site of damage, is focused on the clearance of pathogens. On the contrary, sustained chronic inflammation is associated with neuronal damage and toxicity and is strictly linked to neurodegenerative processes (Guzman-Martinez *et al*, 2019). It is likely that different phenotypes of microglia act over time in both physiological and pathological inflammatory responses and, in the latter case, the proportion of each phenotype may also vary throughout the disease (Tang & Le, 2016). The dichotomized classification in a pro-inflammatory and anti-inflammatory phenotype does not reflect the complex reality and the several microglia phenotypes, that should be considered to belong to a multi-varied spectrum rather than being two definite populations (see **Figure 6**).

Astrocytes are the most representative cells in the brain, contributing to brain homeostasis and neuronal support (Colombo & Farina, 2016). They support neurons by providing energy metabolites and regulating extracellular ionic balance, modulating synaptic activity and neurotrophic factors production, regulating BBB permeability, favoring or limiting the infiltration of other inflammatory cells (Kwon & Koh, 2020). Astrocytes strongly participate in the function of BBB. They have specialized end-feet structures associated with the endothelial cells of the BBB, which regulate fluid and electrolyte passage throughout the high-density expression of water and ionic channel, such as the Aquaporin-4 (AQP4). These channels are involved in water transport and serve as adhesion molecules in leukocytes infiltration during inflammatory responses (Ikeshima-Kataoka, 2016). The astrocytes end-feet expression of the AQP4 and other water-channel molecules influences BBB permeability and has been related to the development of a pro-inflammatory state (Mizee & de Vries, 2013). In addition, several

astrocytes factors may influence BBB permeability. Both the glial-derived neurotrophic factor and the fibroblast growth factor enhance the barrier by stimulating the production of tight junction (Colombo & Farina, 2016). Conversely, astrocytes may release factors increasing BBB permeability and disruption, such as vascular endothelial growth factors, matrix metalloproteinases, endothelin, and nitric oxide in a pathologic state (Michinaga & Koyama, 2019). Astrocytes mediate scar formation, which exerts several beneficial functions, including neuronal protection, BBB repair, and isolation of CNS inflammation, but also negative consequences when exacerbating inflammation and hampering synaptic rearrangement and axon growth (Sofroniew, 2015). Like microglia, astrocytes can be activated by various types of insults and can provide a neuroprotective response, switching off neuroinflammation and stimulating tissue repair, or promoting inflammation, neuronal damage, and death. Two main astrocytes subpopulations have been well characterized: the pro-inflammatory phenotype (A1), producing neuroinflammatory factors such as IL-1a, TNF-α, and complement protein (C)1q, and the immune-modulatory phenotype (A2), which play neuroprotective functions by producing neurotrophic factors and anti-inflammatory cytokines, such as IL-4, IL-10, TGF-β (Liddelow *et al*, 2017). However, multiple phenotypes can be expressed depending on the environmental balance. Astrogliosis's beneficial or detrimental effect is again related to the type of stimulus, the specific disease and its phase, and the involved molecular pathway. Several pathways can mediate neuroprotective astrocytes' response. The glycoprotein (GP)130 is a ubiquitous membrane signal transducer for several cytokines, including IL-6, IL-11, and IL-27 (Ernst & Jenkins, 2004). Stimulation of the GP130 may results in the activation of the intracellular SHP2/Ras/ERK cascade or the STAT 1 and 3. These pathways are essentially neuroprotective by stimulating astrogliosis and scar formation (Wanner *et al*, 2013). In response to high levels of IL-10, a cytokine with anti-inflammatory properties, astrocytes secrete TGF-β, an anti-inflammatory agent mediating the inhibition of the NF-κB, reducing microglia activation and extinguishing inflammation (Norden *et al*, 2014). Reactive astrocytes can express high levels of retinoic acid, which can induce endothelial immune quiescence and reduce leucocytes adhesion and infiltration in the site of inflammation, attenuating reactive oxygen species production (Mizee *et al*, 2014). The ubiquitin-modifying protein A20, a regulator of nuclear transcription factors, suppresses NF-κB activation, pro-inflammatory cytokines

production, and CD4+ T cells recruitment, which is an important element in autoimmune-mediated demyelination in the CNS (Liddelow *et al*, 2017). Dopamine, binding the dopamine D2 receptor expressed by astrocytes, activates the alphaβ-crystallin pathway with anti-inflammatory properties, protecting nigral dopaminergic neurons (Kwon & Koh, 2020). On the contrary, astrocytes can secrete pro-inflammatory factors in response to specific triggers and a specific context. As for microglia, astrocytes exposition to PAMPs, such as LPS, induces activation and a shift to the A1, pro-inflammatory, cytotoxic phenotype with potentially detrimental effects (Liddelow & Barres, 2017). A key modulator of astroglia pro-inflammatory responses is the NF-κB intracellular signaling, which favors neuroinflammation through the upregulation of several pro-inflammatory cytokines, chemokines, and cell-adhesion molecules, stimulating astrogliosis (Kwon & Koh, 2020). The pro-inflammatory cytokine IL-1β stimulates astrocyte production of the vasoactive endothelial growth factor, favoring BBB disruption increasing permeability and lymphocyte infiltration, leading to edema and excitotoxicity (Sofroniew, 2015). The astroglia chemokine C-C Motif Chemokine Ligand-2 exerts pro-inflammatory effects, enhancing M1 microglia activation and leukocytes recruitment and infiltration, stimulating cytotoxicity and axonal damage (Kwon & Koh, 2020). However, similarly to microglia, the pro-inflammatory and the anti-inflammatory state represent only part of the possible astrocyte activities, which should be considered in the context of a multifaced continuum, whose imbalanced regulation paves the way for neuroinflammatory deleterious effects (Liddelow & Barres, 2017).

Oligodendrocytes are the cells designed to produce the myelin sheaths around axons, which allow rapid electrical impulse transmission between neurons. They participate in neurons support and aid regenerative processes. Oligodendrocytes are essential for axonal structural organization. They favor the clustering of sodium channels at the nodes of Ranvier and potassium channels at the juxtaparanodes. In addition, they provide products of glycolysis for axonal metabolism (Duncan *et al*, 2021). Thus, alterations or deficits in oligodendrocytes support results in axon increased vulnerability and loss.

Natural Killer (NK) cells are innate effector lymphocytes acting as a cytotoxic player against malignant cells. In neuroinflammatory responses, they can clean pathological protein aggregates and cellular debris and modulate antigen-presenting cell activities (Earls & Lee, 2020). For example, by interacting with microglia and astrocytes, NK can

suppress the Th17 cells, attenuating inflammatory response (Hao *et al*, 2010). In addition, when stimulated by IL-15 produced by the subventricular zone neural stem cells, they can regulate neuronal repair following brain inflammation (Liu *et al*, 2016).

Mast cells significantly participate in the production of inflammatory molecules. For example, they regulate BBB permeability by producing histamine and modulating the recruitment of other peripheral cells involved in neuroinflammatory responses. In addition, they participate in neurogenesis by producing serotonin and IL-6 and stimulate neuroprotection by secreting neuroprotective cytokines such as IL-1β (Hendriksen *et al*, 2017).

The complement system is a part of the innate immunity involved in recognizing pathogens and favoring phagocytosis and chemotaxis. Complement molecules exert a fundamental role in facilitating interaction between innate and adaptive immunity, thus modulating lymphocytes activity and facilitating antigen presentation (Comi & Tondo, 2017).

The T-lymphocytes primarily mediate adaptive immunity in the CNS. Again, the environment and the levels of several cytokines and chemokines influence the expression of anti-inflammatory or pro-inflammatory phenotypes. Type 2 helper lymphocytes (Th2) and T regulatory (Tregs) cells are involved in anti-inflammatory and regulatory activities, while Type 1 (Th1) and Type 17 (Th17) cells are essentially pro-inflammatory. The expansion of Tregs induces an anti-inflammatory state by reducing the production of pro-inflammatory cytokines and stimulating phagocytic activity of cellular debris and protein aggregates (Comi & Tondo, 2017). B lymphocytes also can participate in both protective and deleterious neuroinflammatory responses. B cells' preeminent role in neuroinflammation has become evident in autoimmune CNS disorders with important neurodegenerative components. It is the case of Multiple Sclerosis, where anti CD20 therapies directed against B lymphocytes expressing the membrane receptor CD20 are effective in contrasting the disease (Stephenson *et al*, 2018).

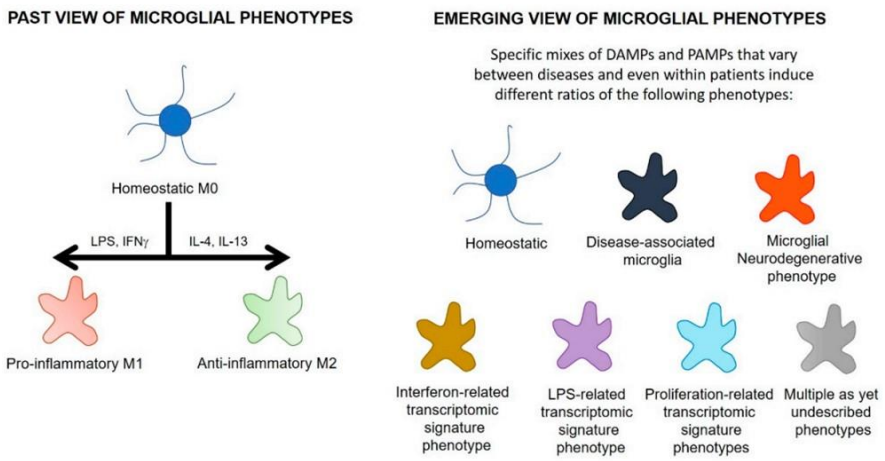


Figure 6. Schematic representation of the earliest and current classifications of microglia phenotypes (source: Werry et al., 2019)**.**

Aging and neuroinflammation

The term "senescence" indicates the progressive and physiological decline of cell functions. Senescence involves all biological processes, including the immune system, which becomes gradually less responsive against harmful stimuli. Immune-senescence also affects the CNS and promotes neuronal dysfunction (Di Benedetto *et al*, 2017). Aging in CNS is associated with reducing neurogenesis, synaptic dysfunction, increased BBB permeability, immune cells alteration. Overall, aging-related changes induce a pro-inflammatory state with deleterious consequences, both in the CNS and at the systemic level, particularly a progressive reduction of neurotrophic factors and shooting down neuroplasticity (Di Benedetto *et al*, 2017). Aging-related changes reflect on immune cells, causing modifications in cells morphology and activity. Senescence induces in microglia a state of altered responsiveness. Microglia are less prompt to detect environmental changes due to a reduction in the activity of the "sensome" for endogenous ligands, while the identification of external pathogens seems to be increased, thus favoring the pro-inflammatory state (Hickman *et al*, 2013). A peculiar microglia phenotype results abundant in the hippocampus, amygdala, hypothalamus, and cerebral cortex during aging, characterized by signs of oxidative stress. These "dark microglia" are more active than

normal microglia, encircling axons and dendrites and favoring an abnormal synaptic remodeling (Bisht *et al*, 2016). Altered microglia are insensitive to negative feedback and further exaggerate neuroinflammatory responses. Chronic neuroinflammation is associated with BBB permeability alteration, which in turn favors leucocytes infiltration of the brain parenchyma, feeding microglia activation and stimulating a low-grade brain inflammation (Di Benedetto *et al*, 2017). In fact, an increased pathological inflammatory state characterizes aged brains compared with younger ones, and the pro-inflammatory environment produces behavioral changes such as anorexia, hypersomnia, lethargy, and deficits in cognitive and motor function (Dilger & Johnson, 2008). In response to the environmental stimuli, microglia shift from the M2 to the M1 phenotype, and activated microglia and astrocytes produce pro-inflammatory cytokines, including IL-1 β, IL-6, and TNF, fostering the pro-inflammatory environment (Di Benedetto *et al*, 2017). The pro-inflammatory state makes the aged brain extremely sensitive to stressors, producing exaggerated responses in the presence of deleterious stimuli or stress, with clinical impact and impairing functional recovery (Sparkman & Johnson, 2008). All the factors contributing to the maintenance of this low-grade chronic pro-inflammatory state facilitate learning impairment, memory loss, and cognitive decline (Chen *et al*, 2016b).

Neuroinflammation in neurodegenerative diseases

Remarkable evidence sustains the primary role of neuroinflammation in neurodegenerative diseases, including AD, PD, and ALS. The chronic activation of neuroinflammatory cells produces detrimental effects, inhibiting tissue repair and promoting tissue damage leading to neurodegenerative processes. **Figure 7** is a schematic representation of the possible link between neuroinflammation and neurodegeneration in proteinopathies. The pathological deposition of misfolded proteins characterizes neurodegenerative disorders. The specific proteinopathy can trigger damage signals or can act as DAMP itself, activating microglia and astrocytes. The glial cells, in turn, produce neurotoxic factors altering protein processing and enhancing misfolded protein deposition. This scheme has received confirmation, especially in AD, PD, and ALS (Golde *et al*, 2013).

In AD, the amyloid-β deposition in the senile plaques and the neurofibrillary tangle formation, due to hyperphosphorylated tau protein, represent the hallmarks of the disease. In addition, the most recognized risk factor for AD is age, which can be associated with neuritic plaques and tauopathy even in the absence of cognitive decline. Besides these pathological hallmarks, both innate and adaptive immune responses have been reported in AD, representing a key player in the process leading to neurodegeneration (Leng & Edison, 2021). Pre-clinical models showed that AD progression is associated with changes in microglia phenotypes, accompanied by the upregulation of known genes associated with AD, such as the apolipoprotein E (*APOE*) and the triggering receptor expressed on myeloid cells 2 (*TREM2*) (Leng & Edison, 2021). APOE is a multifunctional protein with crucial roles in lipid metabolism and is involved in amyloid-β clearance, aggregation, and metabolism. APOE is encoded by three alleles: *ε2*, *ε3*, and *ε4*. The *APOE ε4* allele is recognized as the strongest genetic risk factor for sporadic AD; conversely, the *APOE ε2* allele is considered the strongest genetic protective factor (Serrano-Pozo *et al*, 2021). TREM is a receptor expressed by several innate immune system cells, mediating phagocytosis and modulating neuroinflammatory responses, and the TREM2 isoform has been associated with an increased risk of developing AD (Gratuze *et al*, 2018). *APOE* and *TREM2* expression is finely regulated and participate in neurodegeneration in several ways. Increased levels of TREM2 have been associated in mouse models of AD with increased phagocytic activity and suppression of inflammation (Lee *et al*, 2018). On the other hand, TREM2 activation may also enhance an APOE-dependent pathway stimulating the production of pro-inflammatory molecules (Krasemann *et al*, 2017). The precise characterization of these molecular pathways will help elaborate potential therapeutic approaches, but caution is needed when translating animal models' findings to the human brain. Activated microglia have been found in AD human brain samples, showing variable spatial distribution related to amyloid-β and tau-tangles deposition (Grabert *et al*, 2016). Amyloid-β deposition seems to be the most important element in evocating a deleterious neuroinflammatory activation throughout several mechanisms. Microglia cells bind to soluble β-amyloid oligomers via cell surface receptors, such as TLRs, and secrete pro-inflammatory molecules including cytokines, chemokines, growth factors, prostaglandins, complement factors, reactive oxygen species, and proteases. All these mediators increase the permeability of the BBB, favoring

leukocytes infiltration of the brain. Local neuroinflammation, in turn, activates the expression of pro-inflammatory genes and stimulates the amyloidogenic pathway, increasing β-amyloid and self-stimulating the amyloid cascade (Guillot-Sestier & Town, 2018). TREM2 is one of the microglia receptors involved in this vicious circle. TREM2 can stimulate phagocytosis of amyloid-β plaques and microglia activation. However, this function can be compromised in pathophysiologic conditions associated with neurodegenerative changes, such as in aging, resulting in increased amyloid-β production and deposition (Streit *et al,* 2004). Dysfunctional microglia have also been associated with tau deposition. Microglia activation can be determined by tau, and in those cases aims to tau deposits clearance, but in dysregulated responses, it results in neuronal damage and tau spread, thus contributing to disease progression (Brelstaff *et al*, 2018). The integrity of the BBB plays an essential role in pathological protein deposition, especially in the formation of the amyloid-β plaques. The receptors for advanced glycation end products (RAGEs) are DAMPs that, similarly to the TLRs, trigger and stimulate neuroinflammatory responses. RAGEs recognize and bind amyloid-β, and this interaction favors BBB disruption and microglia activation, sustaining neuronal damage and hampering a successful amyloid-β clearance (Origlia *et al*, 2010). Astrocytes actively participate in the deleterious inflammatory response. The presence of amyloid-β senile plaques alters important astrocyte functions, including maintaining synaptic integrity, neurotransmitter uptake, and calcium signaling, whose disruption causes neuronal excitotoxicity (González-Reyes *et al*, 2017). Astrocytes contribute to β-amyloid clearance throughout a process mediated by APOE, but their dysregulated activation breaks this mechanism and facilitates amyloid-β spread (González-Reyes *et al*, 2017). However, microglia and astrocytes act in combination in neuroinflammatory responses, driving neurodegeneration. Amyloid-β activates the NF-κB pathway in astrocytes, which secrete the C3 protein, activating microglia, enhancing neuroinflammation (Lian *et al*, 2016). On the other hand, activate microglia produce pro-inflammatory cytokines such as IL-1α and TNF, which stimulate the activation of astrocytes (Liddelow *et al*, 2017). This enhanced cross-talk leads to dysregulated inflammatory responses, damaging neurons. Also, the adaptive immune system may play a role in AD pathogenesis. Animal models showed that the activation of peripheral leukocytes, including T-regulatory cells, natural killer, B cells, and neutrophils, might contribute to a state characterized by diffused reactive

gliosis, which favors neuronal dysfunction and death (Marsh *et al*, 2016). Clinical studies in AD patients using CSF and blood biomarkers and imaging studies of neuroinflammation helped underline the role of neuroinflammatory responses in favoring neurodegenerative changes. Several cytokines, namely IL-1β, IL-6, IL-12, IL-18, TNF-γ, and TGF-β, are over-produced in AD patients than in controls, both in CSF and in blood. However, variability in results in different clinical studies, which are also influenced by the degree of peripheral inflammation, hampers conclusive remarks (Swardfager *et al*, 2010). Several neuroimaging studies have been performed to detect *in vivo* neuroinflammation in AD, targeting molecules overexpressed by activated microglia and astrocytes. These studies confirm that neuroinflammation is associated with neurodegeneration and contributes to the deposition of amyloid-β and tau (Jain *et al*, 2020).

PD is the most common neurodegenerative disease after AD, affecting about 2% of the population aged more than 65 years (McCann *et al*, 2014). As for AD, age is the most important nonmodifiable risk factor. The aggregates of α-synuclein represent the pathological hallmark of the disease, associated with a marked loss of dopaminergic neurons in the substantia nigra, clinically leading to motor disturbances (tremor, rigidity, and bradykinesia) and non-motor symptoms, including autonomic dysfunction, cognitive deficits, sleep and neuropsychiatric disturbances (Brás *et al*, 2020). Several animal studies and human *post-mortem* reports suggested the evidence of activated microglia, astrocytes, and inflammatory molecules in response to α-synuclein aggregation (Nagatsu *et al*, 2000). The deposition of α-synuclein favors the shift from the M2 protective microglia phenotype to the pro-inflammatory M1 phenotype (Zhang *et al*, 2005). Chronic exposure to the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin specifically targeting neurons in the basal ganglia and substantia nigra, causing neuronal loss and PD related symptoms, reduce the expression of the M2 phenotype (Pisanu *et al*, 2014). Astrocytes, like microglia, respond to α-synuclein deposition by producing pro-inflammatory cytokines, including IL-1-β and TNF-α (Tanaka *et al*, 2013). Reactive astrocytes have a different gene expression and change their phenotype, becoming more prone to scar formation (Sofroniew, 2015). Microglia activation mediated by α-synuclein, via the TLRs leads to several pro-inflammatory cytokines that stimulate the A1 shift in astrocytes (Fellner *et al*, 2013). The A1 phenotype is associated with loss of normal

astrocytes activity, neuronal damage, and death, especially in dopaminergic neurons (Liddelow *et al*, 2017). Altered neuroinflammatory responses in PD can also be delineated in other cells than microglia and astrocytes. When activated, Mast-cells release matrix metalloproteinases, whose production is increased in the presence of α-synuclein aggregates, and accelerate neuronal loss. In mouse models of PD, MPTP stimulates the mast cells to produce tryptase and microglia and astrocytes to produce pro-inflammatory factors such as the chemokine ligand 2, which favor chemotaxis of other neuroinflammatory cells sustaining a chronic inflammatory activation (Sandhu & Kulka, 2021). The sustained neuroinflammatory response also induces BBB increased permeability and disruption, favoring peripheral leucocytes infiltration into the brain and adaptive immunity activation. Peripheral inflammation in PD is also testified by the presence of increased cytokine levels, including IL1β, IL2, IL6, IFNγ, and TNFα, in both serum and CSF of PD patients compared with controls, and altered count of CD4+ and CD8+ lymphocytes (Reale *et al*, 2009). IL-1β seems to play a crucial role in neurodegenerative changes associated with PD. In mouse models, chronic overexpression of IL1β in the substantia nigra causes most of the characteristics of PD, including progressive dopaminergic cell death, bradykinesia, and activation of microglia (Ferrari *et al*, 2006), while in human plasma levels of IL-1β were found to be significantly higher in PD patients compared with controls, directly correlating with motor impairment and with plasma α-synuclein levels (Fan *et al*, 2020).

ALS is characterized by progressive degeneration of motor neurons in the brain and the spinal cord. Pathogenic mechanisms include mitochondrial dysfunction, cytoskeletal disruption, reactive oxygen species-mediated toxicity, and neuroinflammatory abnormal responses (Mejzini *et al*, 2019). As in other neurodegenerative diseases, the deposition of misfolded proteins is one of the hallmarks of the disease, leading to progressive damage of the motor neurons. In ALS, the most important aggregates include the superoxide dismutase 1 (SOD1), the TDP-43, and the FUS protein (Guerrero *et al*, 2016). TDP-43 deposition plays a crucial role in ALS neuroinflammatory responses and pathogenesis. Extracellular TDP-43 activates microglia enhancing pro-inflammatory pathways such as the NF-κB, which is involved in motor neurons death (Zhao *et al*, 2015). Also, FUS represents a critical activator of the NF-κB pathway, leading to motor neuron apoptosis induced by TNF (Kia *et al*, 2018). However, most evidence regarding microglia

activation in ALS pathogenesis is related to SOD1. SOD1 mutations involving only neurons are insufficient to precipitate the disease in ALS mouse models, while mutations ubiquitously expressed, thus also involving glial cells, cause a rapidly progressive and fatal disease (Lino *et al*, 2002). In addition, the deletion of SOD1 mutation in motor neurons does not protect from disease progression, which is sustained by the glial mutation; coherently, the deletion of the mutation in the myeloid cells' slow disease progression (Boillée *et al*, 2006). SOD1 mutations induce both microglia and astrocytes activation and overexpression of the IL-1β, which has been related to ALS progression (Meissner *et al*, 2010). SOD1 mutation mice also show altered astrocyte activity, which can differentiate in a pro-inflammatory phenotype overproducing TGF-β, which has been associated with accelerated disease progression (Endo *et al*, 2015). Other gene mutations are associated with neuroinflammation in ALS. Mutations in *TREM2* can compromise microglia phagocytic activity, leading to altered neuroinflammatory responses and promoting motor neurons damage (Cady *et al*, 2014). TGF-β is one of the most studied cytokines as a systemic biomarker in ALS (De Marchi *et al*, 2021). Chronic overproduction of TGF-β may sustain disease progression by favoring neurodegeneration of motor neurons (Peters *et al*, 2017). Coherently, high plasma levels of TGF-β have been reported in ALS patients compared with controls, correlating with disease severity (Duque *et al*, 2020). All innate and adaptive immune cells are potentially involved in neuroinflammation driving neurodegeneration in ALS (De Marchi *et al*, 2021). There is evidence of a role for Natural killer cells in ALS sustained inflammation. Natural killer cells infiltrate the motor cortex and the spinal cord of ALS patients and can directly cause motor neurons death or cause inflammatory dysregulation producing IFNs and reducing Tregs recruitment (Garofalo *et al*, 2020). ALS patients show lower Tregs count, especially at the late disease stage (Beers *et al*, 2017). The number of Th1 and Th17 increases with disease progression, testifying to the contribution of peripheral immunity in participating to clinical impairment (Saresella *et al*, 2013).

In conclusion, several molecular and cellular mechanisms are involved in neuroinflammatory responses in AD, PD, and ALS. **Figure 8** represents neuroinflammatory mechanisms in each disease's, depicting main players and involved pathways. Microglia activation represents a crucial step in neuroinflammatory responses leading to neurodegeneration, but the dynamic regulation of cells and activities still needs

to be underlined entirely. The modulation of neuroinflammation, which affects disease progression and trajectory, might have potential therapeutic effects, and research is focusing on the pre-disease period to test disease-modifying strategies.

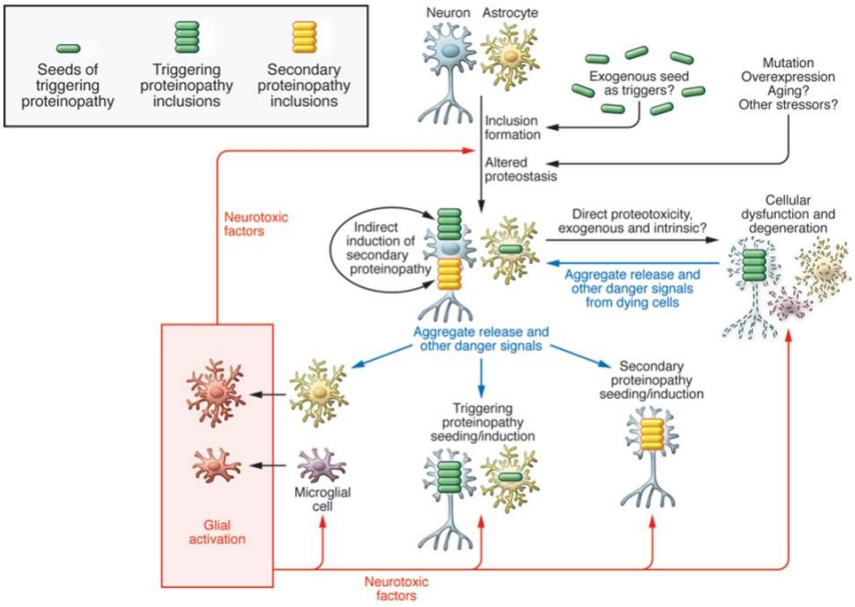


Figure 7. Representation of neuroinflammatory responses generate by pathological misfolded protein deposition in proteinopathies (source: Golde et al., 2013)**.**

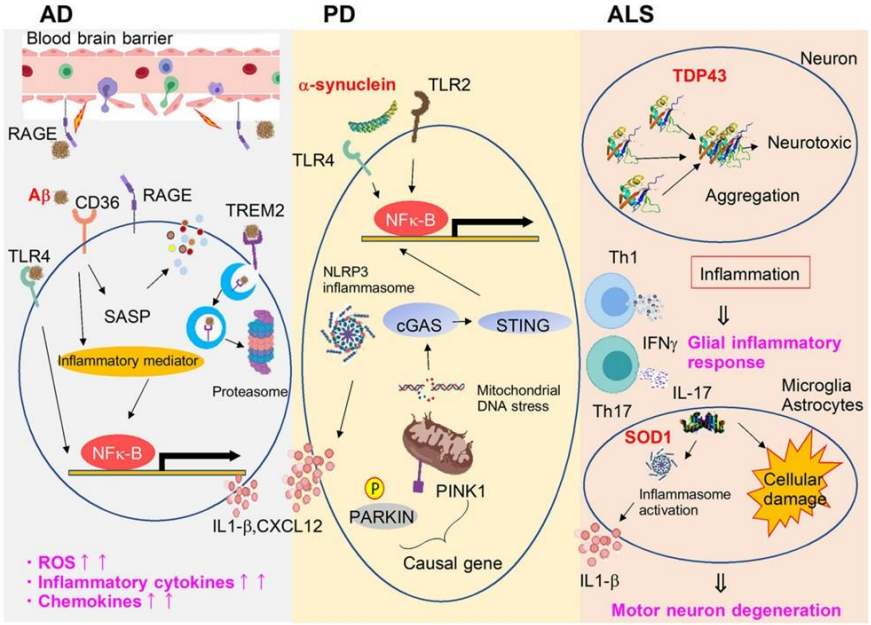


Figure 8. Main neuroinflammatory players and corresponding pathways involved in neurodegenerative changes in Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis (source: Otani et al., 2020)**.**

PET imaging in neurodegenerative diseases

AD is characterized by the histopathological evidence of extracellular amyloid-β plaques and intracellular neurofibrillary tangles containing hyperphosphorylated tau. The hypothetical model on AD pathogenesis states that the pathological alterations occur years before the symptoms' onset, following a sequential order in which misfolded proteins deposition lead to neuronal dysfunction and loss and progressively to clinical decline (Jack Jr *et al*, 2013). Neuroinflammation represents a further player in AD pathogenesis. All these alterations can be detected *in vivo* using biomarkers, which aid the diagnostic workup and potentially improve the development and monitoring of disease-modifying therapeutic approaches (Hansson, 2021). In addition, the possibility to underline biomarker changes in a preclinical phase allows the early identification of the neurodegenerative process, not only in AD but also in other neurodegenerative diseases (Agrawal & Biswas, 2015). PET is a critical tool able to reveal, *in vivo*, several molecular, biochemical, and pathobiological events. These include brain metabolism changes, blow-flow alterations, synaptic abnormalities, neuroreceptor transmission modifications, misfolded protein deposition, and neuroinflammatory responses (Ricci *et al*, 2020). There is full-blown evidence that combining multiple PET imaging modalities improves diagnostic accuracy and prognosis predictive ability, helping to clarify the relationship between different neurodegenerative processes and decisively contributing to the update of therapeutic strategies (Hargreaves & Rabiner, 2014). The last two decades represented a considerable step forward in the field of PET neuroimaging. Significant technical, analytical, and computational advances have been developed (Ricci *et al*, 2020). PET imaging allows the identification of several molecular and biochemical processes targeting them with specific compounds labeled with radio-isotopes. Radioactivity decay of the radio-isotopes can be externally detected, providing a non-invasive, *in vivo* mapping of the distribution of the previously administered compounds and the specific molecular mechanism in which they are involved (Pimlott & Sutherland, 2011). The ^{18}F and the ^{11}C are the most used radio-isotopes in PET imaging studies and for clinical purposes. ^{11}C is used only in highly specialized facilities provided by the cyclotron due to the relatively short half-life of about 20 minutes. For the same reasons, its application is mainly limited to research studies. ^{18}F imaging has more favorable properties for PET analysis, and the half-life of 110 minutes also allows availability in centers lacking

radiochemistry facilities. In addition, tracers labeled with ^{18}F permit longer *in vivo* studies; thus, they are more widely diffused in clinical settings (Zhu *et al*, 2014). Regardless of the used radio-isotopes, PET imaging relies on detecting the radioactivity produced by the radio-labeled compound. After injecting the compound, a variable time is needed to achieve the proper tissue distribution, mainly depending on the tracer and the target. The radioactive isotope targeting the molecular process decays during the scan, emitting a positron annihilated and converging its mass into energy, creating two photons released in opposite directions. These photons can be detected externally by a camera, and the line joining the detected locations passes directly through the point of annihilation. This point is very close to the point of positron emission, giving a reasonable estimation of the position of the radioactive atom in the body. The detector recognizes as many annihilation photons as possible and localizes them along the line the decay occurred. All the detected events are subjected to elaboration through complex mathematical algorithms to be reconstructed into images. These images can be further analyzed using the quantification method for advanced statistical approaches (Cherry & Dahlbom, 2006).

In the last twenty years, the progressive shift from a phenotypical classification of neurodegenerative disorders to biological classification represented an excitant deal for nuclear medicine, reliably providing markers for neuronal injury, synaptic dysfunction, and pathology. However, in clinical practice, patients affected by different underlying neurodegenerative disorders may manifest similar phenotypes. Thus the *in vivo* pathological diagnosis remains a challenge. In this context, PET imaging has become crucial in unrevealing the complex relationship between the manifested clinical phenotype and the underlying pathological process.

PET imaging of brain metabolism

The most widely and best-validated PET tracer is the [^{18}F]FDG, introduced in brain imaging 40 years ago (Phelps *et al*, 1979). Brain activity is essentially based on glucose consumption, and FDG is a glucose analog, which is trapped into neurons after intracellular phosphorylation by the enzyme hexokinase (Sokoloff *et al*, 1977). Cells with higher metabolic activity expend more FDG, which can be used as a direct index of

synaptic activity. Variations in synaptic activity are associated with corresponding oxygen and glucose consumption modification, and synaptic dysfunction is associated with a regional reduction in glucose metabolism (Kato *et al*, 2016). The ability to detect neuronal dysfunction, which precedes neuronal loss for a considerable period, makes [^{18}F]FDG-PET one of the leading biomarkers in identifying early signs of neurodegeneration, with the best sensitivity, specificity and accuracy reached when applying standardized and validated quantification methods, clear cutoff values, and observer-independent analyses (Perani *et al*, 2014b).

[^{18}F]FDG-PET can reveal the hypometabolism pattern, reflecting the extent and the topography of neuronal dysfunction, thus the endo-phenotype of neuronal damage and the corresponding clinical deficit (Capitanio *et al*, 2019). Each dementia syndrome presents a specific regional distribution of neurodegenerative changes, and [^{18}F]FDG-PET underlines the corresponding hypometabolism pattern aiding differential diagnosis, although some overlap may exist. As such, [^{18}F]FDG-PET differentiates with high accuracy patients affected by dementia and cognitively unimpaired subject, and it helps distinguish AD from other dementia, such as DLB and FTD (Herholz *et al*, 2002; Rabinovici *et al*, 2011; Kantarci *et al*, 2021). In addition, it may show specific hypometabolism patterns in subsyndromes within the same pathological spectrum (Laforce Jr *et al*, 2018).

Typical AD cases are characterized by hypometabolism recognizable in temporoparietal cortices, precuneus, and posterior cingulate cortex (Mosconi *et al*, 2008). Several variants in the AD hypometabolism pattern have been reported. The typical temporoparietal hypometabolism pattern is more severe in EOAD than in LOAD, depending on the more severe pathology and more significant clinical impairment (Kim *et al*, 2005). PCA is characterized by early impairment of visuospatial and visuo-perceptive abilities, and [^{18}F]FDG-PET shows corresponding hypometabolism in occipital and posterior parietal cortices (Rosenbloom *et al*, 2011). The logopenic variant PPA shows a prevalent left perisylvian hypometabolism, reflecting language impairment (Madhavan *et al*, 2013). Finally, the frontal AD variant manifests, besides the typical AD pattern, more severe hypometabolism involving the frontal lobes and reflecting the clinical impairment in executive functions and behavior (Woodward *et al*, 2015). Both in typical and atypical AD cases, it is noteworthy the association between the topographic

distribution of brain hypometabolism and the corresponding cognitive impairment, thus [^{18}F]FDG-PET can also help quantify the clinical impact of neurodegeneration and in staging the disease (Perani, 2013; Ou *et al*, 2019). **Figure 9** represents specific hypometabolism patterns in AD subtypes.

A specific hypometabolism pattern is detectable also in other neurodegenerative dementia, aiding differential diagnosis in uncertain cases. In the FTLD spectrum, several hypometabolism patterns can be detected (Ricci *et al*, 2020). The bvFTD is most frequent FTLD subsyndromes, clinically characterized by the executive and neuropsychiatric disturbances, and presents a typical hypometabolism pattern involving the frontal and the anterior temporal regions, the medial temporal structures, the insula, and subcortical regions such as basal ganglia and thalamus (Jeong *et al*, 2005). The clinical differential diagnosis between different types of PPA may be clinically challenging and include the two language forms of FTD, the SD and the non-fluent variant PPA, and the logopenic variant associated with AD (Tippett, 2020). [^{18}F]FDG-PET can be extremely useful in these cases, showing hypometabolism involving the anterior temporal lobe in SD, often with left asymmetric dominance, and the prevalent asymmetric involvement of the fronto-insular, perisylvian and supplementary motor areas in the non-fluent variant PPA (Matías-Guiu *et al*, 2015; Rabinovici *et al*, 2008). Specific hypometabolism patterns can be detected in FTLD atypical parkinsonian syndromes, where consistent clinical overlap can be present, complicating diagnostic workup. In PSP, hypometabolism involves mainly the frontal regions, motor and premotor cortices, the basal ganglia (caudate nucleus), and the brainstem, while in CBD [^{18}F]FDG-PET imaging can reveal asymmetric (contralateral to the clinically most affected body side) hypometabolism in basal ganglia and parietal-frontal cortices (Hellwig *et al*, 2012). Similar to AD, [^{18}F]FDG-PET can be useful in FTD in revealing the correlation between neurodegenerative changes associated with clinical impairment (Cerami *et al*, 2015b), in evaluating disease progression (Diehl *et al*, 2004) and potentially in evaluating response to therapy (Chow *et al*, 2011). The correlation between glucose hypometabolism and cognitive impairment is crucial when evaluating patients affected by ALS who may or not experiment cognitive decline. Frontal and prefrontal hypometabolism is associated with ALS-FTD and correlates with cognitive impairment (Canosa *et al*, 2016). In patients sharing the C9Orf72 mutation, the single-subject analysis revealed different patterns consistent with the clinical phenotypes

(Castelnovo *et al*, 2019). However, in pure ALS cases, [^{18}F]FDG-PET's ability to distinguish between different clinical subtypes showed inconclusive results (Sala *et al*, 2019a).

In DLB, the specific pattern of hypometabolism involves mainly the occipital areas, visual association cortices, and the posterior temporoparietal regions (Perneczky *et al*, 2008). [^{18}F]FDG-PET hypometabolism in the occipital cortex, sparing the posterior cingulate cortex, the so-called cingulate sign, is considered a supportive finding for the diagnosis of DLB, showing high sensitivity and specificity (Graff-Radford *et al*, 2020; McKeith *et al*, 2017). While hypometabolism in temporoparietal areas overlaps with the typical AD pattern, occipital lobe hypometabolism represents a distinguishing feature of DLB (Caminiti *et al*, 2019). However, PCA hypometabolism is more complex to distinguish from DLB hypometabolism than AD typical cases (Whitwell *et al*, 2017a). As for AD and FTD, also in DLB, brain hypometabolism correlates with clinical impairment, and the cingulate signs have been associated with cognitive decline and visual hallucinations (Iizuka & Kameyama, 2016).

For its accuracy in discriminating between different patterns of neurodegenerative dementia [^{18}F]FDG-PET is included in the diagnostic criteria of several neurodegenerative disorders, comprising AD, PPA, bvFTD, and DLB (Gorno-Tempini *et al*, 2011; Dubois *et al*, 2014; McKeith *et al*, 2017). Since [^{18}F]FDG-PET can identify metabolic changes indicative of synaptic dysfunction, which precede neuronal loss, it can detect early signs of neurodegeneration before the occurrence of brain atrophy as detected by MRI (Bateman *et al*, 2012; Garibotto *et al*, 2017). Thus, brain metabolism alterations can also be detected in the prodromal phase of dementia (Cerami *et al*, 2015c). [^{18}F]FDG-PET is of utmost importance in delineating disease progression and prognosis, especially in predicting conversion of the MCI condition (Cerami *et al*, 2015c; Caminiti *et al*, 2018). In MCI individuals, the typical AD hypometabolism pattern involving temporoparietal cortices, predicts conversion to AD with an accuracy higher than 90% (Perani *et al*, 2016). On the other hand, a normal [^{18}F]FDG-PET scan excludes actual signs of neurodegeneration being associated with clinical stability over a follow-up of several years (Iaccarino *et al*, 2019).

Based on these evidences, it appears clear that local neuronal dysfunction revealed by regional hypometabolism represents a well-known marker of neurodegenerative

disorders (Capitanio *et al*, 2019). However, neurodegeneration changes can affect both specific brain regions and high-order neural networks (Fornito *et al*, 2015). [^{18}F]FDG-PET is a precious tool also in analyzing long-distance brain network organization, namely metabolic connectivity. The brain connectivity concept relies on the evidence that brain regions are structurally and functionally connected (Pievani *et al*, 2014). Brain connectivity analysis exploits correlations and covariates of specific signals across the brain, including the blood oxygen level-dependent (BOLD) signal in fMRI, the metabolism signal in [^{18}F]FDG-PET, the cortical electrical activity in electroencephalography. The study of brain network connectivity sheds light on the dynamics of pathological changes in neurodegenerative diseases (Fornito *et al*, 2015). Metabolic connectivity refers to the relationship between [^{18}F]FDG-PET signal in different brain regions. It assumes that regions whose glucose consumption is correlated are functionally interconnected (Horwitz *et al*, 1984). Several analytical methods have been developed to analyze this correlation. The most used techniques are the interregional correlation analysis, the principal component analysis and the independent component analysis, the sparse inverse covariance estimation, the graph theory (Yakushev *et al*, 2017). Interregional Correlation Analysis is a voxel-wise method relying on the a priori selection of one or more seeds, whose tracer uptake is extracted and correlated with the rest of the brain voxels (Morbelli *et al*, 2013). The resulting networks have similar features to resting-state networks obtained by fMRI, showing high discrimination properties (Yakushev *et al*, 2017). Principal Component Analysis and Independent Component Analysis are voxel-wise multivariate approaches based on identifying statistically independent components (Sala & Perani, 2019). This method allows the identification of components in a data-driven manner. However, the investigator needs to select meaningful components and discharge statistical noise components. Sparse inverse covariance estimation is a ROIs-based approach allowing the creation of a connectivity matrix and simultaneously imposing sparsity to potentiate statistical robustness. Thus, it can also be applied when included subjects are lower than the selected ROIs (which is not rare in PET studies) (Sala & Perani, 2019). Graph theory develops the sparse inverse covariance estimation estimating the whole brain connection matrix and applying a threshold to the pairwise correlation values, which permits to estimate brain hubs, modules, and global network indices (Yakushev *et al*, 2017). Metabolic connectivity

studies were mainly focused on the AD spectrum, showing reducing metabolic connectivity involving AD-signature regions such as posterior cingulate gyrus and precuneus (Ballarini *et al*, 2016; Herholz *et al*, 2018). These regions are characteristically part of the default mode network, the major resting-state large-scale brain network, including the medial prefrontal cortex, posterior cingulate cortex, left and right inferior parietal lobes, and left and right hippocampi. Accordingly, several functional MRI studies reported default mode network alterations in AD, with the degree of abnormalities correlating with disease progression severity (Zhang *et al*, 2010; Zhou *et al*, 2010). Coherently, the gradual disruption of the default mode network has also been hypothesized in metabolic connectivity studies, using [^{18}F]FDG-PET data in the AD continuum (Pagani *et al*, 2017). The default mode network appears to be particularly important for cognitive integrity in neurogenerative disorders. A disruption in the default mode network has been associated with cognitive impairment development in PD (Spetsieris *et al*, 2015). Other intriguing findings underline the potential role of metabolic connectivity analysis in revealing mechanisms associated with neurodegeneration. Besides decreased connectivity, increased metabolic connectivity has also been reported in AD but associated with reserve proxies such as education and bilingualism (Perani *et al*, 2017; Malpetti *et al*, 2017). In this case, increased metabolic connectivity may represent a compensatory mechanism favored by lifelong protective factors to counteract more severe hypometabolism (Sala & Perani, 2019).

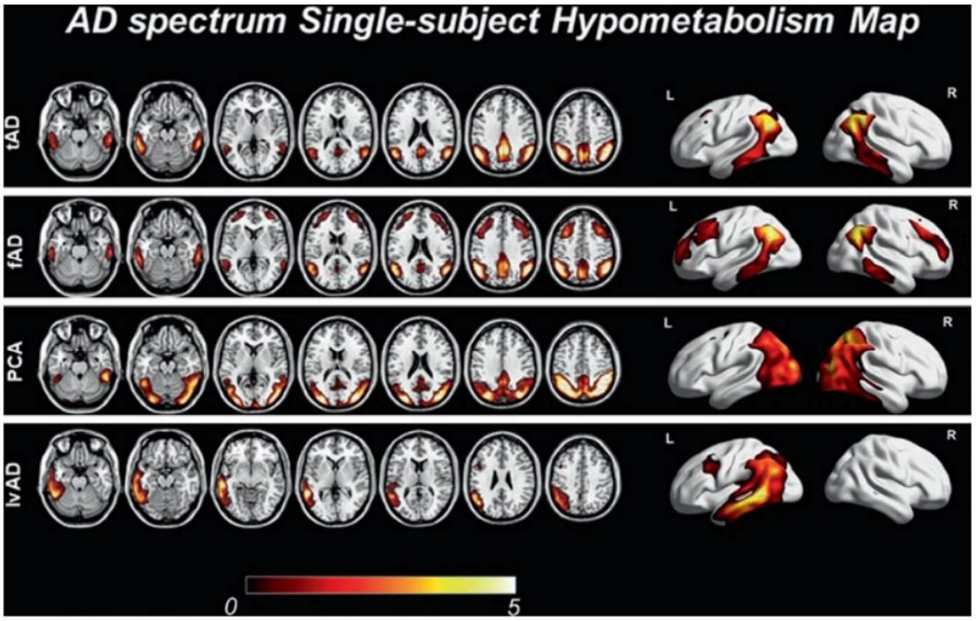


Figure 9. Specific single-subject [^{18}F]FDG-PET hypometabolism patterns in Alzheimer's disease subtypes (source: Perani et al., 2020)**.**

PET imaging of amyloid-β pathology

In the field of PET-imaging of AD pathology in living patients, the crucial starting point was 2004, when Klunk and colleagues published the first in human amyloid-PET study using ^{11}C-labeled Pittsburgh Compound-B ([^{11}C]PiB), and showing that the tracer could discriminate between AD patients and healthy controls (Klunk *et al*, 2004). Since then, amyloid-PET has provided a decisive contribution to the definition of *in vivo* AD pathology (Villemagne *et al*, 2018). The [^{11}C]PiB is currently the most widely used amyloid-PET radiotracer in research settings. In AD patients, [^{11}C]PiB-PET can reveal brain cortical amyloid-β plaques deposition, especially in temporoparietal and frontal regions, which discriminates patients from normal controls, and has been confirmed by *post-mortem* validation (Villemagne *et al*, 2013). [^{11}C]PiB is highly selective and affine to amyloid-β plaques, but it has some technical limitations, including the short half-life of ^{11}C, which hamper its extensive use in clinical centers not provided by a cyclotron unit. For clinical application, the Food and Drug Administration and the European Medicines Agency approved the use of three fluorinated tracers for detecting brain amyloid-β deposition: [^{18}F]Florbetapir, [^{18}F]Florbetaben, and [^{18}F]Flutemetamol (Laforce Jr *et al*,

2018). All these tracers bind to fibrillar amyloid-β plaques and have comparable diagnostic accuracy. **Figure 10** shows an example of amyloid-PET positivity in an AD patient. Their uptake *in vivo* has shown to be highly concordant to *post-mortem* brain tissue pathology (Sabri *et al*, 2015; Ikonomovic *et al*, 2020; Morris *et al*, 2016). The introduction of amyloid-PET imaging allowed to perfect the hypothetical model on AD pathogenesis, revealing the presence of pathological alterations years before the onset of clinical symptoms of dementia. The use of amyloid-β imaging in clinical trials improved selection strategies and stimulated therapeutic approaches (Sperling *et al*, 2014). Beyond uses in research settings, amyloid-PET showed high value in improving clinical diagnostic accuracy due to its ability to detect *in vivo* amyloid-β plaques, and for this reason, it has been included in the guidelines for the AD and prodromal AD diagnosis (McKhann *et al*, 2011; Albert *et al*, 2011). Thus, the earliest objective of amyloid-PET imaging was to reveal brain amyloidosis in patients with a clinical AD diagnosis, aiding in confirming or excluding AD pathology (Rowley *et al*, 2020). In AD, amyloid tracer binding is diffused and elevated mainly in the prefrontal cortex and posterior cingulate cortex, parietal-temporal cortices, and striatum (Clark *et al*, 2011). This pattern of distribution shows minimal intraindividual variation between AD patients. It seems unrelated to the clinical phenotype, to the pattern of neurodegenerative changes, and cognitive decline (Rabinovici *et al*, 2010; Lehmann *et al*, 2013). Amyloid-PET imaging shows its full potential in aiding differential diagnosis between patients in the AD spectrum and non-AD dementia, specifically in atypical AD cases (Wolk *et al*, 2012). Distinguishing AD from FTLD is a fundamental clinical use for amyloid-PET imaging because clinical and anatomic overlap may exist, especially in patients younger than 65 years (Musa *et al*, 2020), but amyloid-β plaques are not evident in the FTLD spectrum. In the most extensive study comparing AD and FTLD patients, amyloid-PET visual rating showed to differentiate patients with about 90% sensitivity and 84% specificity (Rabinovici *et al*, 2011). Amyloid-PET imaging has been used to reveal amyloidosis in individuals with PPA. It easily identifies subjects with the logopenic variant PPA, associated with high cortical amyloid-tracer uptake in more than 90% of cases (Rabinovici *et al*, 2008). The *in vivo* detection of brain amyloidosis has considerable relevance in overt dementia and the prodromal dementia phase. MCI represents a heterogeneous condition, and amyloid-PET imaging is helpful to identify MCI with

underlying AD pathology (Albert *et al*, 2011). In MCI subjects, amyloid-PET positivity is associated with a higher risk of progression to dementia when compared with individuals with normal scans (Wolk *et al*, 2018; Ong *et al*, 2015) and with greater longitudinal cognitive decline (Doraiswamy *et al*, 2014). However, amyloid-β deposition is not exclusive of AD. Amyloid-PET positivity increases with increasing age in AD dementia, non-AD dementia, and even in cognitively normal individuals (Ossenkoppele *et al*, 2015). Furthermore, it is associated with *APOE* status. Thus, evidence suggests that amyloid deposition is an early player in the cascade of neurodegeneration, which is necessary but insufficient to determine cognitive impairment in AD (Villemagne *et al*, 2018). The presence of amyloid-β pathology in a high quote of MCI subjects and unimpaired individuals, together with the lack of clear correlations between cortical amyloid deposition and synaptic dysfunction and the lack of topographical correspondence between regional amyloidosis and the clinical phenotype, confirm this assumption.

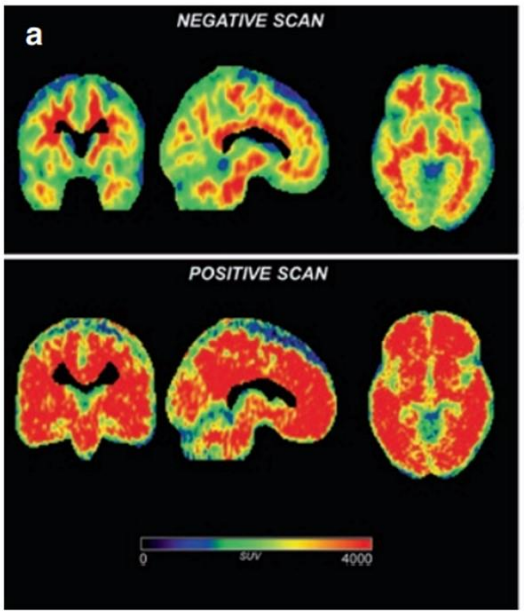


Figure 10. Amyloid-PET positivity in a single Alzheimer's disease patient (source: Perani *et al.*, 2020)**.**

PET imaging of tau pathology

Tau protein is physiologically involved in microtubules stabilization and axonal trafficking. In several neurodegenerative disorders, including AD, tau protein deposition alters axonal transport and neuronal stability, facilitating progressive neuronal dysfunction and loss (Medeiros *et al*, 2011). The research in the field of tau-PET imaging studies overcame several criticisms over the past fifteen years. Firstly, tau aggregates are intracellular. Thus, they are a challenging target for PET radiotracers which need to pass the BBB and the neuronal plasma membrane. In addition, tau may have several protein conformation and isoforms, which modify the binding site for potential tau-PET tracers (Bischof *et al*, 2017). However, the same tau deposit can be associated with different phenotypes and, conversely, the same clinical manifestation may be linked to several tau conformations (Medeiros *et al*, 2011). The first radiotracer that binds neurofibrillary tangles was the [^{18}F]FDDNP, which also has an affinity for amyloid-β plaques. It has been used as a non-selective tau-PET tracer in several early AD studies and other neurodegenerative conditions (Shin *et al*, 2011). Later, several new compounds were developed and studied mainly in AD and MCI: the [^{11}C]PBB3, the THK aryquinoline derivatives, the pyrido-indole derivative [^{18}F]AV-1451 (Chandra *et al*, 2019). The main limitation in using THK compounds is the recognized off-target binding to the Monoamine oxidase-B (MAO-B), enzymes widely distributed in the human brain (Harada *et al*, 2018). The most widely used tau-PET radiotracer is the [^{18}F]AV-1451, also called flortaucipir, binding with high affinity to the 3R/4R tau isoform typical in AD (Marquié *et al*, 2015). Flortaucipir shares with other first-generation tracers the same limitations, including off-target binding, but at a lower degree (Jang *et al*, 2018). More recently, second-generation tau-PET tracers have been developed with a higher affinity to tau tangle than the prototypes and showing low or absent off-target binding (Bischof *et al*, 2017). [^{18}F]MK-6240 and [^{18}F]PI-2620 are two promising tau tracers showing high affinity to tau deposits in AD patients, showing poor off-target binding (Lohith *et al*, 2019; Kroth *et al*, 2019).

Flortaucipir imaging studies confirmed the ability to assess the distribution and extent of tau pathology in AD, which is characterized by high tracer binding in the hippocampus and temporal lobe and in parietal, occipital, and frontal cortices (Passamonti *et al*, 2017).

The pattern of tau deposition as detected by flortaucipir-PET identifies AD patients with fair accuracy (Maass *et al*, 2017), aiding differential diagnosis patients affected by other neurodegenerative tauopathies, such as PSP (Passamonti *et al*, 2017). Brain tau deposition as detected by tau-PET imaging follows the Braak staging, which identifies a progressive topographical involvement of brain regions starting from the trans entorhinal region, followed by hippocampal and limbic involvement and, at the latest stage, affecting neocortical regions (Braak & Braak, 1991). Thus tau-PET imaging can be used to stage AD (Schwarz *et al*, 2016). The significant advance in the clinical setting provided by tau-PET imaging is strictly linked to the relationship between regional tau deposition and cognitive impairment. Several tau-PET imaging studies employing different tau tracers showed in AD patients that tau-deposition directly correlated with cognitive decline severity or impairment in specific cognitive domains, such as memory (Pontecorvo *et al*, 2017; Ossenkoppele *et al*, 2016; Whitwell *et al*, 2018). At difference with amyloid plaques deposition, cortical tau deposition is highly correlated with synaptic dysfunction, neurodegenerative changes, and grey matter atrophy (Das *et al*, 2018; Xia *et al*, 2017; van Eimeren *et al*, 2017; Ossenkoppele *et al*, 2016). These findings align with *post-mortem* results showing that neurofibrillary tangles distribution and not amyloid status correlates with antemortem cognitive status (Nelson *et al*, 2012). In addition, the progressive tau deposition correlates with the progression of neurodegenerative changes and cognitive decline (Schöll *et al*, 2016). Consequently, tau-PET is sensitive in discriminating specific patterns in AD clinical variants (Xia *et al*, 2017; Dronse *et al*, 2017; Ossenkoppele *et al*, 2016; Phillips *et al*, 2018). Thus, PCA is characterized by high flortaucipir uptake in occipital cortices, while logopenic variant of AD shows increased cortical uptake in temporal and frontal regions (Tetzloff *et al*, 2018). EOAD, whose clinical decline is faster and more significant than LOAD, shows higher tau tracer binding in neocortical regions (Schöll *et al*, 2017). **Figure 11** represents typical tau-PET deposition patterns in AD clinical variants. Tau-PET studies' evidence led to reconsider the amyloid cascade hypothesis since tau deposition can be present regardless of the amyloid status, for instance, in medial temporal lobes (Pontecorvo *et al*, 2017). However, tau spreading seems to be associated with, and accelerated by, amyloid deposition (Schöll *et al*, 2016; Pontecorvo *et al*, 2017).

Several interesting results have been reported using tau-PET in the FTLD spectrum, but results impose caution due to the uncertain affinity of the current tau tracer for 4R and 3R tau isoform tauopathies. Nevertheless, flortaucipir can show a different topographical distribution in AD and bvFTD, with the latter characterized by higher subcortical, frontal and insular binding (Cho *et al*, 2018). Also, PPAs may show a different pattern of tau distribution, with the non-fluent variant PPA manifesting higher prefrontal tau tracer uptake and SD having elevated uptake in the anteromedial temporal lobe (Josephs *et al*, 2018). However, the high flortaucipir uptake in SD, which is pathologically characterized by TDP-43 deposits, motivates concerns about the specificity of the tau-PET signal (Tsai *et al*, 2019). The presence of off-target binding may explain these results. Analogously, flortaucipir and other tau-PET tracers can show subcortical distribution, involving mainly basal ganglia and thalamus, in PSP and CBD (Passamonti *et al*, 2017; Ishiki *et al*, 2017; Cho *et al*, 2017; Marquié *et al*, 2017). Again, off-target binding to substances highly distributed in those regions, such as neuromelanin and monoamine oxidases B, impedes obtaining conclusive results (Vermeiren *et al*, 2018).

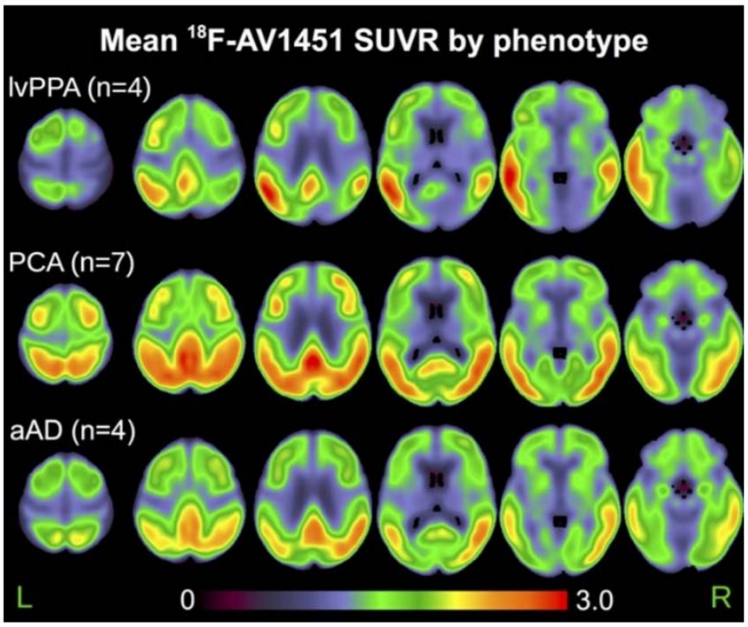


Figure 11. Tau-PET deposition patterns in Alzheimer's disease variants (source: Phillips *et al.*, 2018)**.**

PET imaging of neuroinflammation

Besides the deposition of misfolded proteins, a joint pathological event in neurodegenerative disease is the presence of chronic, dysregulated, and deleterious neuroinflammatory responses (Ransohoff, 2016). *Post-mortem*, *in vitro*, and animal model studies consistently reported the activation of neuroimmune cells strictly linked to the deposition of amyloid-β, tau, and α-synuclein (Brelstaff *et al*, 2018; Lee *et al*, 2013; Zhang *et al*, 2005). In the last two decades, a large part of research in neurodegenerative disease has focused on the mechanisms linking neuroinflammation and neurodegeneration to open new frontiers for developing successful treatments (Du *et al*, 2017). Microglia and astrocytes represent the principal neuroinflammatory CNS cells, capable of multiple protective or deleterious roles, depending on environmental stimuli (Comi & Tondo, 2017). Clinical studies suggested that microglia and astrocytes activation represent an early phenomenon in the neurodegenerative cascade in several brain disorders. However, their precise role and the mechanisms that modulate the neuroinflammatory response in different phases of the disease are still far from being clarified entirely (Du *et al*, 2017). PET imaging of neuroinflammation represents a crucial tool in visualizing neuroinflammatory responses in living patients affected by different neurodegenerative disorders (Narayanaswami *et al*, 2018). Activated microglia and astrocytes overexpress the 18 kDa translocator protein (TSPO), an intracellular protein formerly known as peripheral benzodiazepine receptor (Papadopoulos *et al*, 2006). TSPO is a protein located on the outer mitochondrial membrane involved in steroid synthesis and mitochondrial functions, modulating membrane permeability and calcium-mediated signaling; in addition, it modulates cells proliferation and apoptosis (Gatliff & Campanella, 2012). TSPO multiple roles and functions are not entirely delineated, but its overexpression during neuroinflammatory responses makes this protein a reliable marker of neuroinflammation (Narayanaswami *et al*, 2018). Unfortunately, TSPO lacks specificity, being expressed not only by microglia and astrocytes but also by endothelial cells and, to a lesser extent, by neurons (Gatliff & Campanella, 2012).

[^{11}C]PK11195 is the prototypical TSPO-PET tracer, able to *in vivo* measure TSPO brain levels. It is the first identified TSPO tracer and, to date, the most widely used for tracking neuroinflammation by PET (Cerami *et al*, 2017). However, low brain

permeability, high non-specific binding, and low signal-to-noise ratio limit its use (Jain *et al*, 2020). Several second-generation tracers have been developed to improve signal specificity. However, all second-generation tracers share substantial interindividual variability due to the presence of a polymorphism in the TSPO gene, which affects the binding properties of the TSPO, resulting in high, poor, and mixed affinity binding. The lack of TSPO signal in low-affinity binders obliges genetic testing before the enrollment of subjects (Kreisl *et al*, 2018). Third-generation tracers, insensible to the TSPO polymorphism, are currently under evaluation (Jain *et al*, 2020).

Despite kinetic and methodological limitations, TSPO-PET has been primarily used to underline *in vivo* microglia activation in several neurodegenerative conditions. Since the earliest study, [^{11}C]PK11195-PET showed high TSPO expression in AD patients in several cortical areas, including temporoparietal cortices and the posterior cingulate, able to discriminate patients from controls (Cagnin *et al*, 2001). Microglia activation in AD patients was confirmed in several cortical regions, including prefrontal, parietal, anterior cingulate, and occipital cortex, using different TSPO tracers (Edison *et al*, 2008; Yokokura *et al*, 2017; Passamonti *et al*, 2018). In addition, several studies reported an inverse relation between microglia activation and cognitive function (Yokokura *et al*, 2017; Passamonti *et al*, 2018; Edison *et al*, 2008; Fan *et al*, 2015a). Coherently, the evidence of an inverse relationship between microglial activation and brain glucose metabolism suggests that chronic neuroinflammation may contribute to neurodegeneration in AD (Fan *et al*, 2015b). **Figure 12** reveals different patterns of microglia activation distribution in AD variants. The contribution of microglia to neuronal damage is evident both at the regional and long-distance network organization level (Passamonti *et al*, 2019). Microglia activation measured by TSPO-PET has also been reported in MCI subjects, but with contrasting results. TSPO overexpression seems associated with amyloid-β deposition (Parbo *et al*, 2017). Nevertheless, few studies using [^{18}C]PK11195 or second-generation tracers failed to detect neuroinflammation in MCI (Schuitemaker *et al*, 2013; Kreisl *et al*, 2013). These inconclusive results may partially be explained by the small and heterogeneous samples often included in PET studies of neuroinflammation and methodological differences in assessing TSPO signal (Turkheimer *et al*, 2015).

Microglia activation plays an essential role also in other neurodegenerative conditions. In FTLD, increased [^{11}C]PK11195 binding has been reported in frontal and temporal regions and subcortical structures, thus reflecting the typical topographic distribution of synaptic dysfunction characterizing this condition (Cagnin *et al*, 2004). The same pattern of microglia activation can be detected in the pre-symptomatic carriers of the MAPT mutation (Bevan-Jones *et al*, 2019). These significant findings suggest the possibility of revealing neuroinflammation as a potential therapeutic target before the symptom's onset. A specific topographical distribution of microglia activation can be detected in PSP, involving the basal ganglia, midbrain, frontal lobe, and cerebellum (Passamonti *et al*, 2018), and in CBD, involving basal ganglia and frontoparietal regions contralaterally to the clinical more affected side (Henkel *et al*, 2004).

Pathological deposition of α-synuclein stimulates microglia activation, as shown by *post-mortem* studies (Imamura *et al*, 2003). Therefore, *in vivo* imaging of neuroinflammation revealed microglia activation in PD, DLB, and MSA. In PD, TSPO overexpression mainly involves subcortical structures, including basal ganglia and brainstem, and frontotemporal cortices (Kang *et al*, 2018), and correlates with motor impairment (Ouchi *et al*, 2005). In DLB, higher TSPO tracer uptake can be detected in the cerebellum and association cortices than in PD patients (Iannaccone *et al*, 2013), showing a correlation with cognitive impairment, supporting the hypothesis that microglia drive neurodegeneration (Surendranathan *et al*, 2018). The degree of neuroinflammatory response also correlates with cognitive impairment and hypometabolism in PD dementia (Edison *et al*, 2013). Microglia activation has been reported in MSA, in cortical and subcortical regions, but without a clear correlation with clinical impairment (Kübler *et al*, 2019). These findings underline that further and longitudinal investigations are needed to clarify the role of microglia activation in α-synucleinopathies.

Other neurodegenerative conditions showed evidence of microglia activation in different phases of the disease. Microglia activation in frontal cortices and midbrain has been revealed using [^{11}C]PK11195 and [^{11}C]PBR28 in ALS patients (Turner *et al*, 2004; Corcia *et al*, 2012). The degree of TSPO expression again correlated with disease severity and structural signs of neuronal damage, as detected by MRI (Zürcher *et al*, 2015; Alshikho *et al*, 2018, 2016). In Huntington's disease, an autosomal dominant

neurodegenerative disease characterized by chorea, parkinsonism, psychiatric symptoms, and dementia, symptomatic mutation carrier manifest microglia activation in cortical and subcortical structures, which correlate with motor dysfunction (Pavese *et al*, 2006). Most importantly, microglia activation can also be detected in pre-symptomatic mutation carriers, confirming the role of neuroinflammation in participating in neurodegenerative changes (Politis *et al*, 2011, 2015).

Astrocytes, together with microglia, are responsible for the overexpression of TSPO during neuroinflammatory responses. Therefore, TSPO imaging cannot distinguish the two immune cell populations when both are activated. Astrocytes activation also induces increased levels of MAO-B, which can lead to neurotoxic effects via glutamate-excitotoxicity, as shown in mouse models of AD (Jo *et al*, 2014). [^{11}C]Deuterium-l-deprenyl (DED) is a PET tracer binding the MAO-B, thus representing a marker of astrocytosis. *Post-mortem* studies showed a topographical distribution of astrocytosis corresponding to the Braak staging in the AD brain (Gulyás *et al*, 2011), and astrocytes activation has been reported, *in vivo*, in AD and ALS (Johansson *et al*, 2007; Rodriguez-Vieitez *et al*, 2016b). Again, the most promising results in this context derive from studies in prodromal and preclinical phases of AD dementia. Increased [^{11}C]DED binding has been reported in MCI individuals with evidence of amyloid-PET positivity compared with negative ones (Carter *et al*, 2012). A study in autosomal dominant AD carriers reported an early peak of astrocytes activation, with a subsequent decline, along the trajectory to dementia (Rodriguez-Vieitez *et al*, 2016a). It is also reported an inverse correlation between the astrocytes activation and brain metabolism, supporting the hypothesis that neuroinflammation may be implicated in the early stage of neurodegeneration (Carter *et al*, 2019).

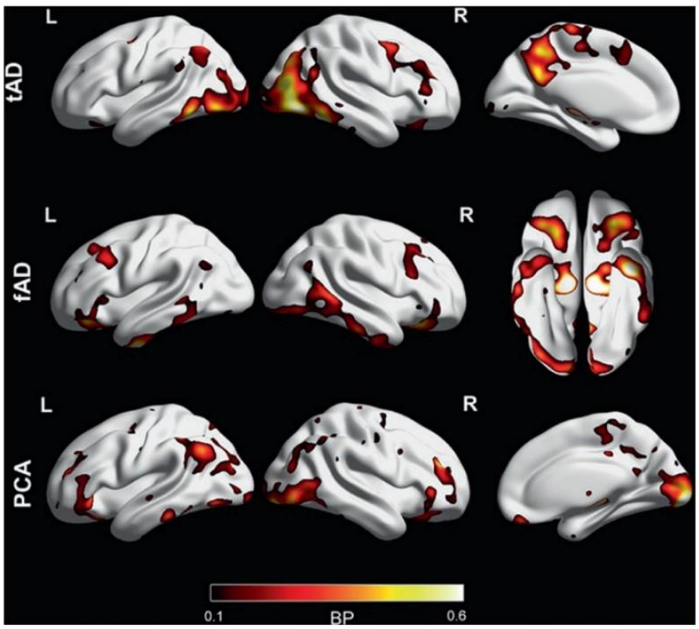


Figure 12. Topographical distribution of TSPO overexpression in Alzheimer's disease clinical variants (source: Perani *et al.*, 2020)**.**

AIM OF THE WORK

This Ph.D. program aimed to evaluate the role of clinical, structural and molecular biomarkers in supporting diagnosis and predicting prognosis in individuals along the dementia continuum. The premises of this dissertation rely on the evidence that early detection of biomarker alterations is crucial to correctly select candidates for clinical trials and in the evaluation of disease-modifying therapies.

The dissertation is divided into two sections.

Section 1 discusses the role of brain metabolism changes in preclinical and prodromal dementia phases and the correlations with structural MRI data, CSF measures, and clinical data. It includes two reviews (Review 1 and Review 2), summarizing the role of univariate and multivariate PET imaging approaches in neurodegenerative diseases, and three studies. Data used in these studies derived from two Italian multicenter projects, namely the "Network-AD project" and the "Interceptor Project", collecting multiple biomarkers in individuals in the dementia continuum. We firstly analyzed brain [^{18}F]FDG-PET and structural MRI in a large population of MCI to provide a cross-sectional framework of the baseline features of subjects at risk for developing AD (Study 1). We then applied the validated SPM single-subject method in MCI cohorts to identify subjects with a favorable prognosis, quantifying the accuracy of [^{18}F]FDG-PET in predicting stability in comparison with CSF biomarkers (Study 2, published). We further extended [^{18}F]FDG-PET analysis to the preclinical dementia phase to identify specific disease hypometabolism patterns and correlate metabolism data with amyloid pathology and neuropsychological characterization (Study 3).

Section 2 discusses the role of neuroinflammation in different phases of neurodegenerative dementias. It is introduced by a review on specific neuroinflammatory mechanisms mediated by the receptor tyrosine kinases, involved in both neuronal development neurodegenerative changes. Two studies are presented in section 2. First, microglia activation has been evaluated in EOAD patients, and the relationship with brain glucose metabolism has been analyzed by studying brain network connectivity (Study 4, published). Second, similar methods were used to detect neuroinflammation in a sample of MCI subjects, to test whether microglia activation was associated with metabolism changes already in the prodromal dementia phase (Study 5, published).

RESULTS

Part 1. PET molecular imaging of neurodegeneration, pathology and relationships with clinical manifestations and prognosis in preclinical and prodromal dementia phases

Review 1. PET imaging in dementia conditions (Perani et al., PET and SPECT in Neurology, 2020)

The growing availability of PET neuroimaging techniques for assessing brain function, molecular mechanisms, and neuropathology has opened a new diagnostic and therapeutic approach to neurodegenerative diseases. The following discussion is based on the published review "PET imaging in dementia conditions" (Perani *et al*, 2020), aiming to highlight each technique's corresponding strengths and weaknesses and emphasize methodological challenges and future perspectives. **Figure 13** summarizes PET neuroimaging evidence (neurodegeneration, amyloid, tau and neuroinflammation PET) in dementia spectra, with color gradients indicating magnitude of alteration, namely, the combination of number of reported evidence and consistency.

[^{18}F]FDG-PET as a marker of neurodegeneration has shown to be precious in diagnosis, evaluating prognosis, staging, and monitoring disease progression (Capitanio *et al*, 2019). One of the central potentials of [^{18}F]FDG-PET imaging, which relies on the identification of hypometabolism regions, that is, brain areas affected by synaptic dysfunction, consists of revealing disease-specific patterns of neurodegeneration (McKhann *et al*, 2011; Gorno-Tempini *et al*, 2011; Armstrong *et al*, 2013; Rascovsky *et al*, 2011; McKeith *et al*, 2017; Albert *et al*, 2011). Consistent evidence suggests the diagnostic utility of [^{18}F]FDG-PET in differentiating AD from FTD and AD from DLB, while literature provides less evidence related to the differentiation of AD from vascular dementia and pseudodementia. However, [^{18}F]FDG-PET is indicated and provides essential information in every circumstance where the clinical picture is unclear (Nestor *et al*, 2018). [^{18}F]FDG-PET signal analysis critically influences the technique's diagnostic accuracy (Iaccarino *et al*, 2017). The adoption of standardized methodologies ensures uniformity and reliability of results, and several parametric methods have been developed

to provide quantification or semi-quantification measurements of the metabolic signal. The Statistical Parametric Mapping (SPM) allows statistical comparisons of available images at the voxel level, providing maps of brain differences among patients or between patients and normative data (Friston, 1994). The single-subject analysis method, a procedure developed at the IRCCS San Raffaele Hospital, Milan, Italy, permits comparing the single-subject metabolism map and a large dataset of normal controls (N = 112), providing a subject-specific metabolism pattern map (Perani *et al*, 2014a; Della Rosa *et al*, 2014). This method has been validated to identify the disease-specific pattern of hypometabolism indicative of different neurodegenerative diseases (Perani *et al*, 2016). At the single-subject level, [^{18}F]FDG-PET underlines, in AD patients, the typical temporoparietal, posterior cingulate, and precuneus hypometabolism pattern, which can differentiate AD from non-AD dementia with 94% sensitivity and 86% specificity. Therefore, detecting a specific hypometabolism pattern crucially improves the accuracy of the clinical diagnosis (Perani *et al*, 2016). The diagnostic accuracy can be even higher when considering other neurodegenerative dementia than AD, reaching 94% accuracy in comparing FTLD and non-FTLD and between DLB and non-DLB forms (Perani *et al*, 2016). Besides the ability to discriminate between different disease spectra, [^{18}F]FDG-PET can detect specific hypometabolism patterns within the same disease spectrum. In FTLD, the single-subject analysis revealed two main variants of bvFTD, namely the frontal and the temporal-limbic. In the frontal AD variant, hypometabolism is mainly detected in the prefrontal cortex, either symmetrically or asymmetrically, and correlates with executive and language deficits. In the temporal-limbic variant, hypometabolism regions include the medial temporal lobes and limbic structures, correlating with poor performances in encoding and recall on long-term memory tasks (Cerami *et al*, 2015b). The clinical-functional relationship may be underlined in the PPA spectrum. Subjects with the semantic variant of PPA may manifest a predominant left or right involvement of the temporal regions. The right semantic variant of PPA hypometabolism predominantly involves the temporal poles, the inferior and middle temporal regions, the insula, the anterior cingulate regions, and the orbitofrontal-cortices, and it is correlated with the clinical development of loss of memory for words and prosopagnosia (Iaccarino *et al*, 2015). The high value of [^{18}F]FDG-PET in diagnostic workup is evident also in atypical parkinsonism. The single-subject analysis revealed a disease-specific

hypometabolism pattern in DLB patients consistently involving temporoparietal and occipital regions. The comparison of the specific DLB hypometabolism pattern with AD and PD dementia patterns resulted in a highly great discriminative power, with an overall accuracy higher than 90% (Caminiti *et al*, 2019).

Most importantly, [^{18}F]FDG-PET improves diagnostic and prognostic evaluation of subjects in the prodromal phase of dementia, with important implications for the development of clinical trials. Brain hypometabolism as detected by [^{18}F]FDG-PET is highly accurate in predicting conversion from MCI to dementia, including not only AD dementia but also other neurodegenerative etiologies, performing better than other biomarkers such as CSF and brain MRI (Perani *et al*, 2016; Caminiti *et al*, 2018; Prestia *et al*, 2013; Dukart *et al*, 2016). In addition, it is worthy of note the exclusionary role of [^{18}F]FDG-PET, since the absence of hypometabolic brain regions testifies the absence of actual signs of neurodegeneration, resulting in a favorable prognosis regardless of the amyloid status (Iaccarino *et al*, 2019). The high prognostic value of [^{18}F]FDG-PET is crucial for supporting the clinician's disease monitoring and choosing the best therapeutic approach. Consistently, some considerations are needed when considering accuracy in design and planning clinical trials, whose participants' selection strategies rely essentially on the amyloid positivity. Amyloid deposition can also be detected in cognitively unimpaired individuals (Chételat *et al*, 2013), and no significant or uncertain relationship between amyloid load and cognition has been repeatedly reported (Marchant *et al*, 2012). Therefore, the inclusion of [^{18}F]FDG-PET as a screening tool for candidates in clinical trials should be considered due to the ability of this technique to detect neurodegenerative changes indicative for AD or other dementia even in the MCI phase. As a marker of neurodegeneration, [^{18}F]FDG-PET would optimize trial effectiveness, reducing the number of recruited individuals not in the AD continuum, positively impacting the trial results, and potentially avoiding the exposure to side effects of healthy individuals. In summary, [^{18}F]FDG-PET has a remarkable ability to underline the typical temporoparietal AD hypometabolism pattern already in the MCI condition, a high value in predicting outcome, and a significant negative predictive role at the single-subject level. For all these reasons [^{18}F]FDG-PET is recommended to evaluate individuals with MCI with suspicion of underlining AD pathology, and it should be considered a marker of neurodegeneration in enrolling candidates for clinical trials (Chételat *et al*, 2020).

The great potential in using [^{18}F]FDG-PET to reveal neurodegenerative changes cannot disguise some criticisms, especially in clinical settings, where the employment of quantification methods is not extremely common. The qualitative interpretation of brain metabolism maps, the visual inspection, significantly affect sensitivity and specificity of [^{18}F]FDG-PET, depending on the clinicians' experience and lacking objective cutoff between normal and pathological findings (Perani *et al*, 2014b). In 2015 a Cochrane Database systematic review collected and analyzed 14 studies (including a total of 421 participants) aiming to determine the diagnostic accuracy of the [^{18}F]FDG-PET for predicting progression to AD or other types of dementia in MCI individuals. The analysis concluded for the absence of current evidence supporting the routine use of the [^{18}F]FDG-PET in clinical practice in MCI subjects. The result is related to the lack of defined pathological thresholds and the extreme variability in specificity values, resulting in an estimated sensitivity of 76% and median specificity of 82% (Smailagic *et al*, 2015). These findings are mainly due to methodological differences across studies. A subsequent review aimed to clarify the role of [^{18}F]FDG-PET in predicting AD in MCI subjects compared to structural MRI and perfusion single-photon emission computed tomography. Although both [^{18}F]FDG-PET and MRI effectively predict AD dementia in MCI condition, the review highlighted the extreme variability in metrics, samples, and outcomes which produced discordant results (A Sanchez-Catasus *et al*, 2017). Thus, there is a need to employ standardized and validated quantification methods to provide reliable and trustworthy results in research studies and clinical settings.

Amyloid-PET is considered a crucial diagnostic tool in AD dementia workup (Dubois *et al*, 2014). Several working groups have proposed criteria for the appropriate use of amyloid-PET in the clinical setting (Minoshima *et al*, 2016; Guerra *et al*, 2015; Johnson *et al*, 2013a). Amyloid-PET imaging should be performed in patients with confirmed cognitive impairment when the etiology is still unclear after a comprehensive evaluation performed by a clinician expert in evaluating dementia and cognitive decline (Minoshima *et al*, 2016). In addition, amyloid-PET is recommended when knowing the amyloid status of the patient may improve diagnostic accuracy and therapeutic management (Guerra *et al*, 2015). Amyloid-PET should be performed to disentangle differential diagnosis between AD and FTD or between AD and vascular dementia. Moreover, it may help in MCI when AD is the primary suspected etiology, but there is an atypical presentation or

in case of symptoms' onset before 65 years (Johnson *et al*, 2013b). Conversely, amyloid-PET should not be performed when the patients have typical AD features and typical AD onset, for determining the severity of dementia, or if the subjects are asymptomatic carrying the *APOE ε4* or with a family history of AD (Guerra *et al*, 2015). As reflected by the provided guidelines, the use of amyloid-PET in specific clinical scenarios is crucial. A large multicenter study, with more than 10000 participants included in the analysis, evaluated the role of amyloid PET, used according to the appropriate-use criteria, in changing patients' management, either MCI or patients with dementia. Amyloid-PET positivity was found in 55% of MCI and 70% of patients with dementia. In this large cohort, the use of amyloid-PET was associated with changes in management in more than 60% of the entire sample (Rabinovici *et al*, 2019). Worthy of note, amyloid-PET positivity is associated, in MCI subjects, with a higher risk to convert to AD dementia, while a normal cortical amyloid load is associated with long-term stability (Ma *et al*, 2014). Both cortical amyloid-β deposition and CSF outcome measures are strictly convergent and similar predictors of conversion (Hansson *et al*, 2018). In an early study using [^{11}C]-PiB-PET, in a population including 31 MCI subjects, 55% showed amyloid-PET positivity and 82% of these converted to AD during a 3-year follow-up, while only one of the 14 amyloid-PET negative MCI progressed to dementia (Okello *et al*, 2009). In a subsequent study in a larger sample including AD patients, MCI and healthy controls and employing [^{18}F]Florbetapir-PET, amyloid-PET positivity was associated in MCI and healthy controls with more significant clinical worsening (Doraiswamy *et al*, 2014). Further studies confirmed the ability of amyloid-PET in predicting conversion to AD in MCI individuals (Ciarmiello *et al*, 2019; Frings *et al*, 2018).

In the last decades, the development of monoclonal antibodies targeting amyloid-β as promising therapeutic agents for AD led to the inclusion of amyloid-PET as an effective screening tool for candidates at the early stage of dementia (Sevigny *et al*, 2016). However, there are several limitations in the use of amyloid-PET in both clinical and research. The pathological amyloid load can be detected by amyloid-PET in almost all AD patients, but cognitively normal elderly may present falsely positive scans (Suppiah *et al*, 2019). In cognitively normal individuals aged more than 70 years, the incidence of amyloid PET positivity is approximately 13% per year (Jack *et al*, 2013). The evidence of amyloid-PET positivity in the older adult with normal cognition is the main limitation

of this diagnostic tool. The rate of amyloid-PET positivity increases with age, ranging from 10% to 44% in individuals with normal cognition in the age between 50 and 90 years (Jansen *et al*, 2015). Another major limitation is the low specificity of amyloid-PET in evaluating patients with non-AD dementia, especially in elderly patients, which may have an abnormal amyloid load. Brain amyloidosis is not an exclusive feature of AD dementia. It can be present in other neurodegenerative conditions, including CAA, DLB, vascular dementia, and FTLD, increasing in aged individuals and *APOE ε4* carriers (Ossenkoppele *et al*, 2015). Amyloid-PET is often positive in DLB patients and in CAA (Seo *et al*, 2017; Petrou *et al*, 2015). In addition, amyloid-β deposition can be seen in atypical AD and in FTLD, especially in patients with older age (Whitwell *et al*, 2020). When evaluating patients within the AD spectrum, amyloid-PET fails in underlying interindividual differences (Dronse *et al*, 2017). Furthermore, although amyloid positive individuals show worse longitudinal cognitive decline than amyloid negative subjects, several studies showed absent or poor correlation between amyloid deposition and neurodegenerative changes (either brain hypometabolism or atrophy) and cognitive decline (Koivunen *et al*, 2011; Altmann *et al*, 2015). The lack of association between cortical amyloid deposition and neurodegenerative regions can be partly explained by the affinity of amyloid-PET radiotracers, which is high for the fibrillar insoluble amyloid-β plaques but low for the more neurotoxic oligomeric forms (Kayed & Lasagna-Reeves, 2013). In addition, the uncertain association between amyloid deposition and clinical symptoms has led to questioning the amyloid cascade hypothesis, which identifies amyloid-β deposition as the driving causative alteration in AD pathogenesis (Selkoe & Hardy, 2016). The need for reconsidering the role of amyloidopathy was also raised about the failure of several clinical trials for AD, where the reduction of amyloid plaques could be reached but without showing significant effects on clinical symptoms or without positively impacting on cognitive decline (Sevigny *et al*, 2016). Lastly, amyloid-PET imaging evaluation may adopt qualitative and quantitative analysis, and different methods for quantification, depending on scan type, tracer properties, signal correction, identification of a reference region, the choice of a specific cutoff of normality, which increases variability and uncertainty regarding the precise definition of abnormal amyloid levels (Alongi *et al*, 2021). In the clinical setting, the interpretation of the amyloid-PET scan is essentially based on the visual assessment, rating the scan as either positive or

negative and neglecting the possibility to have an equivocal visual classification (Payoux *et al*, 2015). Different quantification methods for amyloid-PET imaging have been proposed to improve classification accuracy, but further validation is needed to reveal the clinical meaningfulness of regional amyloid distribution. (Alongi *et al*, 2021)

Tau-PET represents one of the most interesting molecular imaging techniques in the field of neurodegeneration. It allows visualizing tau deposition in living patients, reflecting tau distribution as shown by *post-mortem* studies. Tau deposition is related to neurodegenerative changes, thus correlating with brain hypometabolism and atrophy and to clinical decline, being useful in disease diagnosis, prediction of prognosis, and staging (Villemagne *et al*, 2018). Flortaucipir, the most used tau tracer, showed a specific regional distribution depending on the clinical subtypes in different AD presentations. In typical AD cases, dominated by the amnestic decline, flortaucipir distribution involves mainly temporoparietal regions. On the other hand, in the PCA variant, tracer binding is predominant in the occipital and parietal cortices, in the logopenic variant involves the left temporal-parietal cortex, and in the corticobasal syndrome involves the primary and association sensorimotor cortices asymmetrically (Xia *et al*, 2017). Thus, in contrast with amyloid-β imaging, tau-PET is a crucial tool in revealing the topographical distribution of pathological changes and correlations with clinical impairment (Villemagne *et al*, 2018). Notably, the correspondence between topographical tau distribution and neurodegeneration and, consequently, with clinical symptoms, is present in the AD spectrum since the preclinical phase, following a gradient of deposition aiding the clinical classification. Significant abnormal cortical tau deposition has been reported in AD patients and MCI subjects compared with normal controls, and the presence of high tau tracer binding in the inferior temporal gyrus was associated with clinical impairment (Johnson *et al*, 2016). Thus, tau PET imaging, which is strictly related to the p-tau measurement in CSF, can identify AD patients with high accuracy (Mattsson *et al*, 2018). In addition, tau-PET provided interesting findings in cognitively unimpaired individuals, which fed the discussion about the amyloid cascade hypothesis. A recent study investigated longitudinal tau-PET changes in cognitively impaired and cognitively unimpaired participants to determine variables predicting tau deposition. In cognitively unimpaired individuals, a higher rate of tau deposition was associated with baseline abnormal amyloid-PET, while in cognitively impaired subjects, abnormal amyloid-PET,

APOE ε4, and younger age (i.e., EOAD) were predictors of longitudinal higher tau deposition (Jack Jr *et al*, 2020). These findings add important evidence for screening candidates in clinical trials targeting amyloid and tau since amyloid deposition showed to facilitate longitudinal tau accumulation, which is associated with faster cognitive decline (Jack Jr *et al*, 2018b). In conclusion, tau-PET can be considered a valid candidate biomarker to predict AD progression and monitor disease and response to therapy.

Less clear evidence has been reported in other tauopathies than AD. Studies using several tau-PET tracers reported significant tracer uptake in disease-specific regions, such as basal ganglia in PSP (Whitwell *et al*, 2017b), frontoparietal and subcortical regions in CBD (Smith *et al*, 2017), frontotemporal regions in FTD (Spina *et al*, 2017). However, also contrasting results have been reported. High tau-tracer retention has also been reported in patients with the svPPA with TDP-43 pathology in affected regions, namely temporal lobes and insula (Bevan-Jones *et al*, 2018), and limited differences among tauopathies such as bvFTD and CBD have been demonstrated, confirming the unclear sensitivity and specificity of tau-PET in distinguishing tauopathies other than AD (Tsai *et al*, 2019). The lack of selectivity of the current tau-PET tracers in tauopathies represents the main criticism in tau-PET imaging. While flortaucipir demonstrated a high affinity for the 3R/4R AD tauopathy, the affinity for other tau isoforms, namely the 4R, typical for PSP and CBD, or the 3R, which characterizes the Pick disease, has not been validated (Marquié *et al*, 2015). In addition, off-target binding of all currently used tau-PET tracers needs to be considered. Subcortical tau-tracer off-target binding to molecules other than tau deposits, such as neuromelanin, hampers to obtain solid diagnostic results in several tauopathies (Lowe *et al*, 2016). Finally, suitable and reliable quantification approaches are still to be defined, and all these criticisms limit the diagnostic utility of tau-PET, especially in non-AD tauopathies.

PET imaging of neuroinflammation has provided fundamental insights into the *in vivo* characterization of the pathological mechanisms underlying neurodegenerative diseases. TSPO overexpression is a reliable marker of neuroinflammatory responses in general and, specifically, its production is largely due to microglia activation (Jain *et al*, 2020). Since microglia activation seems to be higher in regions affected by neurodegenerative changes, TSPO-PET has been proposed as a useful tool in providing differential diagnosis among neurodegenerative disorders and their subtypes. A study using the [^{11}C]PK11195, the

most validated TSPO tracer, showed a different pattern of microglia activation in AD and PSP, with increased tracer binding in cortical temporal, parietal and occipital regions in AD patients and higher subcortical TSPO expression in PSP patients (Passamonti *et al*, 2018). Another study analyzed the cortical binding of [^{11}C]PBR28, a second generation TSPO tracer, in AD subtypes. While typical AD showed higher tracer uptake in inferior and medio-temporal cortex, PCA patients had higher microglia activation in occipital, posterior parietal and temporal regions (Kreisl *et al*, 2017). In addition, microglia activation seems to correlate with clinical impairment. In AD patients, TSPO overexpression inversely correlate with neuropsychological performances (Kreisl *et al*, 2013). Again, different correlations can be underlined in different neurodegenerative disorders, since TSPO levels in the cuneus/precuneus correlate with memory impairment in AD, while basal ganglia microglia activation correlate with motor impairment in PSP (Passamonti *et al*, 2018). The relationship between microglia activation and clinical impairment is confirmed by the correlations between TSPO overexpression and brain atrophy and hypometabolism (Kreisl *et al*, 2013). Microglia activation may provide information also relating the longitudinal progression of neurodegenerative changes, and especially in AD, where both temporo-parietal tau pathology and anterior temporal TSPO overexpression have shown to predict cognitive decline in demented patients (Malpetti *et al*, 2020). All these findings confirm that TSPO-PET imaging may serve a s a tool in staging diseases, monitor disease progression and possibly monitor response to therapy. Imaging of neuroinflammation has already been used to monitor novel potential disease-modifying therapies indeed. The compound AZD3241 is an inhibitor of myeloperoxidase, an enzyme expressed by microglia generating reactive oxygen species. AZD3241 has been used in a phase 2 trial in PD patients to reduce microglia activation. After eight weeks of treatment, PD patients showed reduced [^{11}C]PBR28 binding in several regions, including the nigrostriatal region (Jucaite *et al*, 2015). Another study tested the ability of the antibiotic minocycline in reducing microglia activation in MSA. While [^{11}C]PK11195-PET showed a longitudinal reduction in microglia activation in the treated group after 48 weeks, no clinical effect on symptoms severity as assessed by clinical motor function was evident, suggesting that reducing microglia activation does not necessarily generate clinical improvement (Dodel *et al*, 2010).

The lack of clinical repercussion in using anti-inflammatory therapies in neurodegenerative disorders confirms some criticism in using TSPO imaging. Current TSPO tracers do not discriminate between microglia activation and astrocytosis nor distinguish between different microglia phenotypes. Different phenotypes of microglia, proinflammatory, and anti-inflammatory may act in different disease phases of the neurodegenerative process. Thus, distinguish between the neuroprotective and the deleterious responses is crucial for evaluating the effectiveness of therapies. Another limitation in the use of TSPO imaging needs to be considered. The widely and heterogeneous expression of the TSPO across the brain hampers identifying a clear reference region. To overcome this limit, clustering methods have been proposed. However, the adoption of different quantification methods and analysis of tracers with different kinetic properties can explain why contrasting results emerged relating the type and the amount of inflammatory response in different neurodegenerative and psychiatric disorders (Turkheimer *et al*, 2015). Other methodological caveats have emerged using TSPO-PET tracers. [^{11}C]PK11195 has a low brain permeability, limiting its entrance to the brain parenchyma. In addition, it has a high non-specific binding to plasma proteins, resulting in a low signal-to-noise ratio and low specificity (Liu *et al*, 2014). A moderate intraindividual reproducibility has also been reported (Jučaite *et al*, 2012). Due to the [^{11}C]PK11195 chemical and kinetical limitations, several second-generation tracers have been developed to improve signal specificity and quality (Fujita *et al*, 2017). All second-generation tracers, however, share a further critical limitation. Since the first studies, both patients and healthy controls showed large interindividual variability in TSPO second-generation tracers binding. Some subjects presented detectable brain signal, while others showed an absence of tracer binding. This significant variability is due to the presence of a polymorphism in the TSPO gene, the rs6971, which affects the binding properties of the TSPO. In brief, three different genotypes can exist with different binding affinity for TSPO tracers, respectively low, mixed, and high-affinity binding (Owen *et al*, 2012). Low-affinity binder individuals show a negligible TSPO-PET signal. Therefore, the enrollment of these subjects in PET studies is pointless, and they are usually excluded. On the other hand, subjects with high and mixed affinity binding may show notable variability in the TSPO signal. Hence a genotype evaluation is mandatory before the enrollment to avoid inaccuracies in the elaboration of results. Third-generation tracers are

currently under evaluation, overcoming the limitation of low signal-to-noise ratio and insensitive to the TSPO rs6971 polymorphism, but their clinical relevance still needs confirmation (Best *et al*, 2019).

In summary, neuroinflammation is a complex response to harmful stimuli, and its exaggerated activation may drive neurodegenerative changes. TSPO imaging revealed *in vivo* microglia activation in several neurodegenerative disorders. However, the potential to aid diagnosis, monitor disease progression, and test new therapeutic strategies need further evaluation.

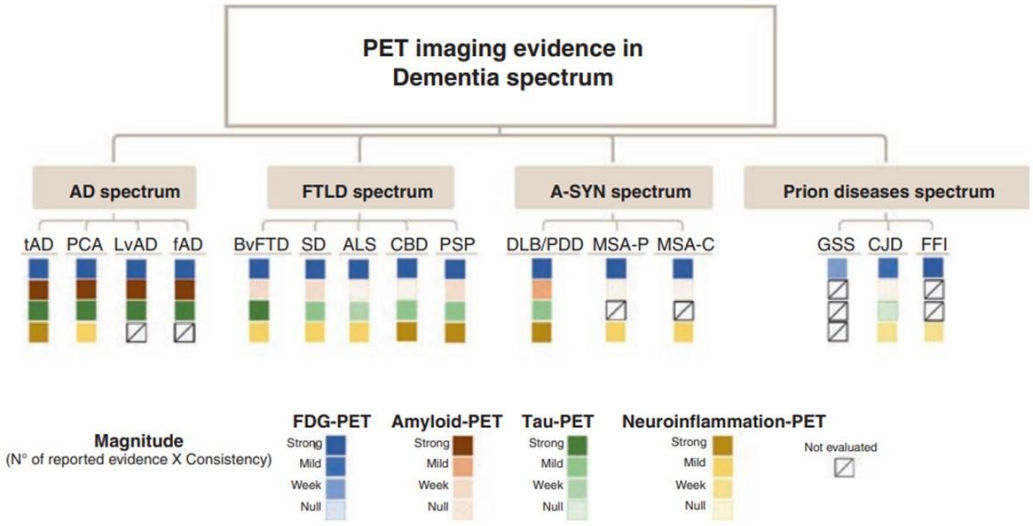


Figure 13. PET neuroimaging evidence of neurodegeneration, amyloid, tau and neuroinflammation in dementia spectra. The color gradient indicates the strength of the evidence, which is higher for all the techniques in the AD spectrum, while in the other dementia spectra, only [^{18}F]FDG-PET and, partly, tau-PET show consistency across studies (source: Perani *et al*., 2020).

Review 2. Brain Molecular Connectivity in Neurodegenerative Conditions (Carli et al., Brain Sci. 2021)

The pathophysiological hypothesis on neurodegeneration considers loco-regional anatomical, molecular, and biochemical changes and the relationship between different brain regions connected in functional and metabolic networks (Palop *et al*, 2006). The connectivity approach initially relied on MRI, namely, functional MRI to assess functional connectivity and diffusion tensor imaging to assess structural connectivity, as well as electroencephalography and magnetoencephalography. More recently, the assessment of brain molecular connectivity employing PET imaging data emerged as a new tool for investigating long-distance network alteration in neurodegenerative diseases (Sala & Perani, 2019). The following discussion is based on the published review "Brain Molecular Connectivity in Neurodegenerative Conditions" (Carli *et al*, 2021), summarizing the most recent advances in brain molecular and metabolic connectivity in neurodegenerative diseases, particularly in the AD spectrum and in the α-synuclein spectrum.

The brain as a complex system can be considered both a "passive" or an "active" player in brain disorders. The neurodegenerative cascade, usually starting with misfolded proteins deposition and precipitating with a series of pathological events, leads to the damage of high-order brain networks, which reverberate to distant brain regions (Sala & Perani, 2019). The pathological process in a specific region promotes the reorganization of long-distance interconnection. The brain, which in this view is considered a passive target of pathology, also has dynamic properties providing a re-modulation of the network in response to neuronal damage (Palop *et al*, 2006). However, recent evidence suggests that neuronal interconnections play an active role in pathological transmission, which follows a prototypical pattern depending on the neurodegenerative substrate (Seeley, 2017). In other words, pathology transmission follows a specific topography in each neurodegenerative disorder, as shown by autoptic studies, and pathological proteins can spread through synapses together with neuronal interconnections in a prion-like manner (Frost & Diamond, 2010). In this vein, the brain network actively facilitates the spread of pathology, representing the topographical constraint in which the pathological alterations can disseminate from the initial site (Zhou *et al*, 2012). Neuroimaging studies confirm

both the passive and active role of brain networks, showing specific connectivity alterations in neurodegenerative disease and, at the same time, delineating topographical diffusion of pathology proceeding with disease progression (Seeley, 2017).

The basic assumption of metabolic connectivity is that brain regions with correlated glucose consumption are functionally connected. This concept has been initially applied in AD. The default mode network is the most analyzed in metabolic connectivity studies in the AD spectrum, since it involves AD signature regions, including the posterior cingulate cortex, the precuneus, temporoparietal cortex, and the medial temporal cortex (Herholz *et al*, 2018). A derangement of the default mode network has been reported in AD patients and MCI subjects (Morbelli *et al*, 2012; Huang *et al*, 2018), with the latter condition showing intermediate alterations between normal cognition and AD (Pagani *et al*, 2017). **Figure 14** represents the typical derangement of the default mode network detectable in AD patients compared with controls. Amyloid-β deposition seems to drive brain network alterations starting from the preclinical phase of dementia, showing a cumulative effect in healthy individuals, MCI, and AD patients (Carbonell *et al*, 2016). Altered metabolic connectivity may show different features according to the clinical phenotype of AD. Typical AD patients manifest a peculiar disconnection between the posterior cingulate and the hippocampus (Herholz *et al*, 2018). EOAD patients have a more extensive brain network disruption, correlating with the severity of dementia (Chung *et al*, 2016). In addition, specific brain network abnormalities correlate with clinical impairment, as shown in EOAD, where neuropsychiatric disturbances were related to dysfunctional changes in brain metabolic connectivity (Ballarini *et al*, 2016). Lastly, metabolic connectivity studies comparing AD and patients affected by other types of dementia, including FTLD and DLB, showed that characteristic alterations in brain network organization could be detected in different neurodegenerative disorders, aiding differential diagnosis (Titov *et al*, 2017; Imai *et al*, 2020). The ability to detect early network dysfunctions, underline specific abnormalities related to the clinical phenotype, and the evidence of a correlation between network alterations and clinical impairment, make metabolic connectivity a promising technique to evaluate subjects in the AD continuum, from the preclinical to the overt dementia phase.

Despite what is evident in the AD spectrum, where specific alterations of the default mode network can be detected already in the early phase, in the α-synuclein spectrum,

considering PD, PDD, and DLB, connectivity derangements can involve multiple large-scale networks (Sala & Perani, 2019). PD patients show brain metabolic connectivity alterations in multiple networks, predominantly in the frontal components of the default mode network, attentional, executive, and motor network (Sala *et al*, 2017). Abnormalities in brain metabolic connectivity reflect clinical impairment, and default mode network is significantly affected in PD patients with MCI than PD patients without cognitive impairment (Spetsieris *et al*, 2015). Posterior cortical damage in PD patients seems to predict cognitive decline (Kehagia *et al*, 2013), while frontal abnormalities have been associated with neuropsychiatric disturbances (Schwartz *et al*, 2019). Consistent evidence of a link between brain network metabolic alterations and clinical impairment has also been reported in DLB patients, showing visuospatial impairment correlating with impaired metabolic connectivity in the primary and associative visual networks, and executive deficits correlating with alterations in frontal metabolic connectivity (Sala *et al*, 2019b). In addition, in DLB patients, impaired connectivity in the default mode network, attention network, and visual network have been associated with the development of visual Hallucinations (Iaccarino *et al*, 2018c; Sala *et al*, 2019b).

Molecular connectivity analysis also allows for tracking specific neurotransmission system alterations starting from the metabolism data of that system. Therefore, [^{18}F]FDG-PET can be used to investigate metabolic connectivity of the dopaminergic neurotransmission system, providing molecular information on the dopaminergic pathway (Sala *et al*, 2017). These details are of particular interest in the study of α-synucleinopathies. For example, both PD and DLB patients show moderate and severe abnormalities in the Nigro-striato-cortical dopaminergic network, in line with striatal dopaminergic studies employing the dopamine-transporter scan (Verger *et al*, 2020). However, the most intriguing findings regard individuals with the isolated rapid-eye-movement sleep behavior disorder, considered the prodromal stage of several α-synucleinopathies (Högl *et al*, 2018). In these cases, only minimal changes in the Nigro-striato-cortical dopaminergic network can be detected. At the same time, other neurotransmission systems (i.e., noradrenergic and cholinergic) are affected by reconfiguration changes, again confirming that the α-synuclein spectrum is characterized by multiple neurotransmission system dysfunctions (Carli *et al*, 2020).

There are some methodological issues related to the brain metabolic connectivity approaches limiting the broad utilization of this technique in clinical and research. The main limitation regards the low spatial resolution of PET imaging, which hampers precise evaluation of small brain nuclei such as midbrain nuclei and the related brain connections (Boellaard, 2009). Furthermore, based on complex covariance patterns, brain network connectivity refers to statistical and mathematical approaches whose biological meaning cannot consistently be demonstrated. In addition, the multivariate analysis methods used in the metabolic connectivity analysis may vary between laboratories and investigators, making challenging the validation of results (Veronese *et al*, 2019). Lastly, to date, the study of metabolic connectivity is permitted only at the group level due to the static nature of PET imaging, while single-subject level analysis would allow obtaining more accurate and predictive data. Despite these well-known limitations, brain metabolic connectivity analysis offers the possibility to underline functional mechanism alterations in neurodegenerative disease. Brain network abnormalities can be detectable in the early stage of neurodegeneration, providing a potential instrument to monitor disease progression and to evaluate response to therapy.

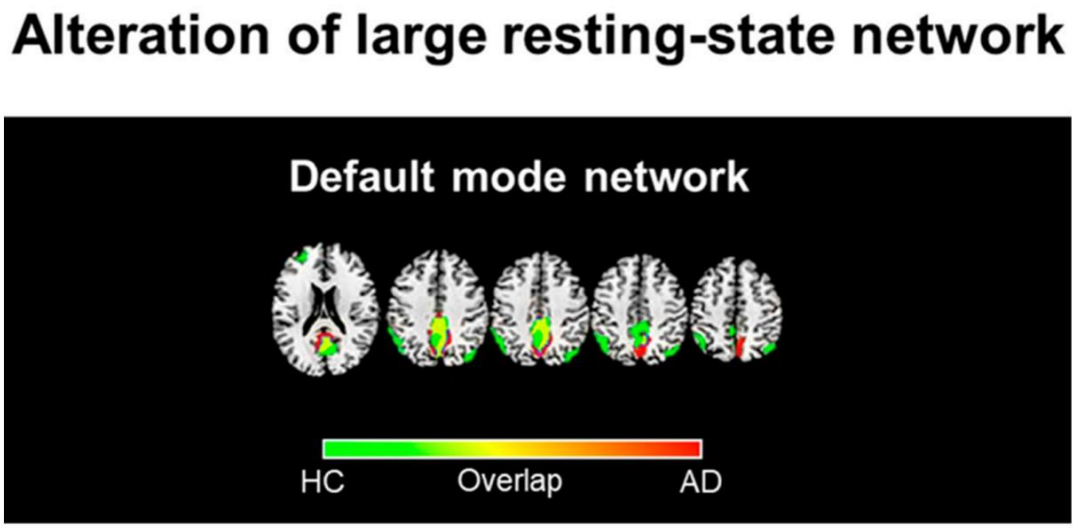


Figure 14. Metabolic connectivity derangements in the default mode network in Alzheimer's disease (source: Carli *et al.*, 2021).

Study 1. Role of imaging biomarkers in the classification of MCI condition as defined by the NIA-AA criteria. Preliminary data analysis

Currently, phase 3 trials experimenting disease-modifying therapy for AD are enrolling patients with mild to moderate AD dementia, subjects with prodromal AD, specifically MCI subjects with biomarker evidence of AD pathology, and preclinical AD, namely normal participants at risk for AD (Cummings *et al*, 2021b). Great expectancies have been placed in the enrollment of MCI subjects as the possible target for therapeutic strategies aiming to modify progression to dementia (Sevigny *et al*, 2016; Sperling *et al*, 2014). MCI is a common condition in the elderly, reaching an overall prevalence of 10.1% in individuals aged 70-74 years, 14.8% for ages 75-79, and 25.2% for ages 80-84 (Petersen *et al*, 2018). Although MCI can be the initial manifestation of AD, it can be due to other neurological, psychiatric or systemic disorders (Huey *et al*, 2013). Consistent evidence showed that MCI subjects have an increased risk of progression to dementia when compared with age-matched unimpaired individuals (Di Carlo *et al*, 2007; Boyle *et al*, 2006). The estimated cumulative incidence for the development of dementia in MCI subjects aged more than 65 years is 14.9% after two years (Petersen *et al*, 2018). However, extreme variability in conversion rate and the prognosis is observed in prodromal AD (Ward *et al*, 2013). In addition, reversion to normal cognition on follow-up has been reported to range from 15 to 50% of individuals with MCI (Thomas *et al*, 2019; Roberts *et al*, 2014; Canevelli *et al*, 2016). Starting from these premises, the MCI candidates' selection for clinical trials should be very accurate to exclude individuals who will never convert to AD, avoid exposure to side effects and resource consumption, and eliminate bias in evaluating beneficial effects.

Among biomarkers used for stratifying subjects according to their risk to convert to dementia, [^{18}F]FDG-PET plays a leading role. The employment of standardized analysis methods for glucose metabolism signal leads to high diagnostic and prognostic accuracy (Perani *et al*, 2016). However, contrasting results have been reported, primarily due to methodological criticisms, heterogeneity in sample selection, or the use of visual assessment (Frings *et al*, 2018). A recent study including more than 300 MCI subjects from the ADNI database examined the predictive role for conversion to dementia of [^{18}F]FDG-PET, amyloid-PET, and non-imaging biomarkers, alone or in combination.

PET imaging was analyzed using voxel-wise principle-components analysis, which allowed to compare predictive values of both methodologies. [^{18}F]FDG-PET outperformed amyloid-PET in prediction accuracy. However, non-imaging variables in combination (including cognitive and functional scales and *APOE* genotype) outperformed both PET imaging techniques. The combination of imaging and non-imaging predictors showed the highest accuracy in predicting conversion to dementia (Blazhenets *et al*, 2020). These findings are in line with previous results. Another study employing multiple biomarkers tested the accuracy of predictors of AD progression in 73 MCI subjects, reporting that the combination of CSF, amyloid-β42 value and [^{18}F]FDG-PET reached the highest positive likelihood ratios. Negativity to all predictors was associated with 100% stability. [^{18}F]FDG-PET was the individual biomarker showing the best performance in predicting MCI progression (Prestia *et al*, 2015).

MRI in an important neuroimaging biomarker able to identify early regional changes associated with the future development of dementia in MCI populations (Whitwell *et al*, 2007). Atrophy starts in the medial temporal lobes and extends to the hippocampus, middle temporal gyrus, and parietal lobe. Medial temporal lobe atrophy, especially with other biomarkers, predicts conversion to dementia with high accuracy (Galluzzi *et al*, 2010; Jack Jr *et al*, 2010b; Trzepacz *et al*, 2014). However, the accuracy in predicting dementia conversion may be influenced by the method of assessing atrophy (Lombardi *et al*, 2020). Validated visual rating scales and quantitative volumetric measures are available (Frisoni *et al*, 2017). Quantitative methods include manual regions of interest approaches and semi-automated and automated segmentation techniques, permitting the identification of normality thresholds (De Francesco *et al*, 2021).

This work presents preliminary data analysis from the Interceptor Project, an Italian multicenter cohort study funded by the Italian Ministry of Health and the Italian Medicines Agency and launched in 2018 (Rossini *et al*, 2019). The study focuses on the MCI condition as defined by the NIA-AA criteria (Albert *et al*, 2011) and has enrolled 400 subjects consecutively diagnosed with MCI at 20 Italian Centers for Cognitive Disorders and Dementia. The project aims to identify a biomarker, or a combination of biomarkers, able to predict with greater accuracy the conversion of MCI to AD dementia after three years of follow-up. In addition, the analysis will include the rate of conversion to dementia other than AD, the rate of stability and reversion and factors associated with

favorable prognosis, and the financial sustainability of the multiple biomarkers approach. The project, lasting 54 months with a six-month follow-up, includes cognitive, neuropsychological, imaging, CSF, genetic, and neurophysiological biomarkers. Specifically, as cognitive and neuropsychological biomarkers, have been included the Mini-Mental State Examination (MMSE) (Folstein *et al*, 1975; Magni *et al*, 1996) and the Free and Cued Selective Reminding Test (FCSRT) (Clerici *et al*, 2017; Frasson *et al*, 2011), associated with an extended neuropsychological evaluation comprising test to evaluate memory, language, visuospatial, executive functions. Imaging biomarkers comprise structural MRI hippocampal volumetry and [^{18}F]FDG-PET analysis. CSF measures include amyloid-β40, amyloid-β40, t-tau, p-tau, and the tau/amyloid-β ratios. All included subjects are tested for the *APOE* genotype, with at least one *APOE ε4* allele as genetic biomarkers. Electroencephalography with functional connectivity analysis has been considered as a neurophysiological biomarker to predict conversion from MCI to AD. See the "Materials and methods" section for further details on the selection of biomarkers, MCI inclusion and exclusion criteria, and data analysis.

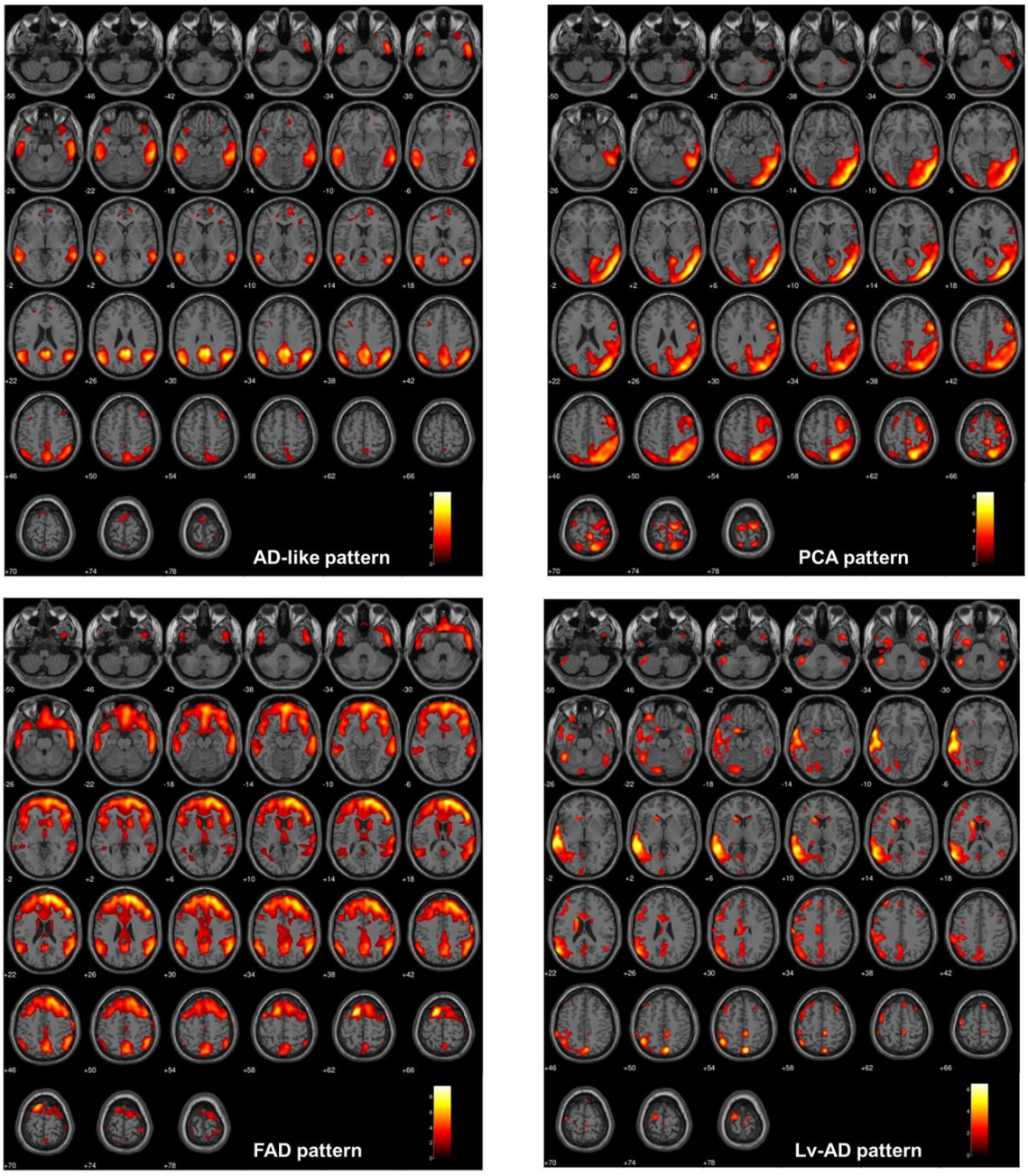
This preliminary work presents [^{18}F]FDG-PET and MRI structural data with volumetric analysis of the hippocampal region. The SPM single-subject method has been applied on the all available [^{18}F]FDG-PET scans, testing each scan for relative hypometabolism through a two-sample t-test (implemented in SPM12) comparing the image on a voxel-by-voxel basis with a large normal database (N = 112) of [^{18}F]-FDG PET images, including age as covariate (Perani *et al*, 2014a). This method has shown high accuracy in predicting conversion from the MCI condition to AD dementia and other neurodegenerative diseases and predicting long-term clinical stability (Caminiti *et al*, 2018; Iaccarino *et al*, 2019). In addition, this method has shown to be valid in analyzing images acquired from different scans (Presotto *et al*, 2018a), thus being particularly useful in handling multicenter studies data. The single-subject method allows obtaining the subject-specific map of brain hypometabolism, which can be visually rated into different patterns: a) normal brain metabolism; b) temporo-parietal hypometabolism, namely AD-like pattern (Perani *et al*, 2016; Cerami *et al*, 2015c); c) hypometabolism in the frontal cortex, specifically frontal-like pattern (Cerami *et al*, 2015c); d) temporo-parietal and occipital hypometabolism, namely DLB-like pattern (Caminiti *et al*, 2017); e) striatal or cerebellar hypometabolism, the MSA-like pattern (Caminiti *et al*, 2017); f) limbic-

predominant or medial-temporal pattern (Cerami *et al*, 2018). **Figure 15** represents single-subject SPM hypometabolism patterns indicative of different underlying etiologies, in the AD and in the non-AD spectrum. A total of 369 [^{18}F]FDG-PET has been analyzed (mean age ± standard deviation 71.44 ± 9.43 years, female/male = 189/180). Eighteen scans were excluded due to artifacts. Of the resulting 351 images, N = 280 (80%) were classified as abnormal hypometabolism patter. The AD-like hypometabolism pattern was present in N = 140/351 MCI subjects (40%). In the AD-like pattern group, most MCI subjects present a typical AD pattern (N = 110, 79%). Among the atypical AD patterns, the most frequent was the PCA (N = 15; 11%), followed by the frontal AD variant (N = 10; 7%) and by the logopenic variant PPA (N = 5; 3%). The non-AD hypometabolism pattern was evident in N = 140/351 (40%) of MCI subjects. Among non-AD hypometabolism patterns, the most frequent was the medial-temporal hypometabolism pattern (limbic-predominant) (N = 58; 41%), followed by the frontal like-pattern (N = 42; 30%), and the DLB (N = 6; 4%) and MSA patterns (N = 5; 4%). Subjects showing multiple cortical and subcortical spots of hypometabolism, as in vascular cognitive impairment, were classified in the cerebrovascular/non-neurodegenerative patter (N = 29; 21%).

Hippocampal volume analysis has been conducted with the FreeSurfer pipeline for brain segmentation (Fischl, 2012). The automated method allows to obtain right and left hippocampal volumes in each individual, which is normalized to the intracranial volume and compared to a normal control database for identifying MCI subjects whose hippocampal volume results is below the fifth percentile. The FreeSurfer segmentation has been applied to the MCI subsample showing an AD-like hypometabolism pattern. Results are presented in **Figure 16**. MRI was available for 120 MCI subjects. Left or right hippocampal atrophy has been detected in N = 38 MCI subjects (32%). Significant hippocampal volume reduction increases with increasing age (**Figure 16**).

Overall, [^{18}F]FDG-PET analysis revealed, in a large sample of MCI, that 40% of the population has an AD-like hypometabolism pattern, while the same percentage shows a non-AD hypometabolism pattern, indicative for e different etiology than AD. In this subsample, a quote corresponding to 16% of the total is represented by limbic-predominant MCI, a condition associated with long-term clinical stability (Cerami *et al*, 2018). The hippocampal volumetry analysis applied to the [^{18}F]FDG-PET AD-like

pattern group shows a high rate of hippocampal atrophy and a significant difference with the normal controls group. The integration of [^{18}F]FDG-PET and MRI data and the longitudinal assessment of MCI subjects included in this study will clarify the two biomarkers' predictive combined value.



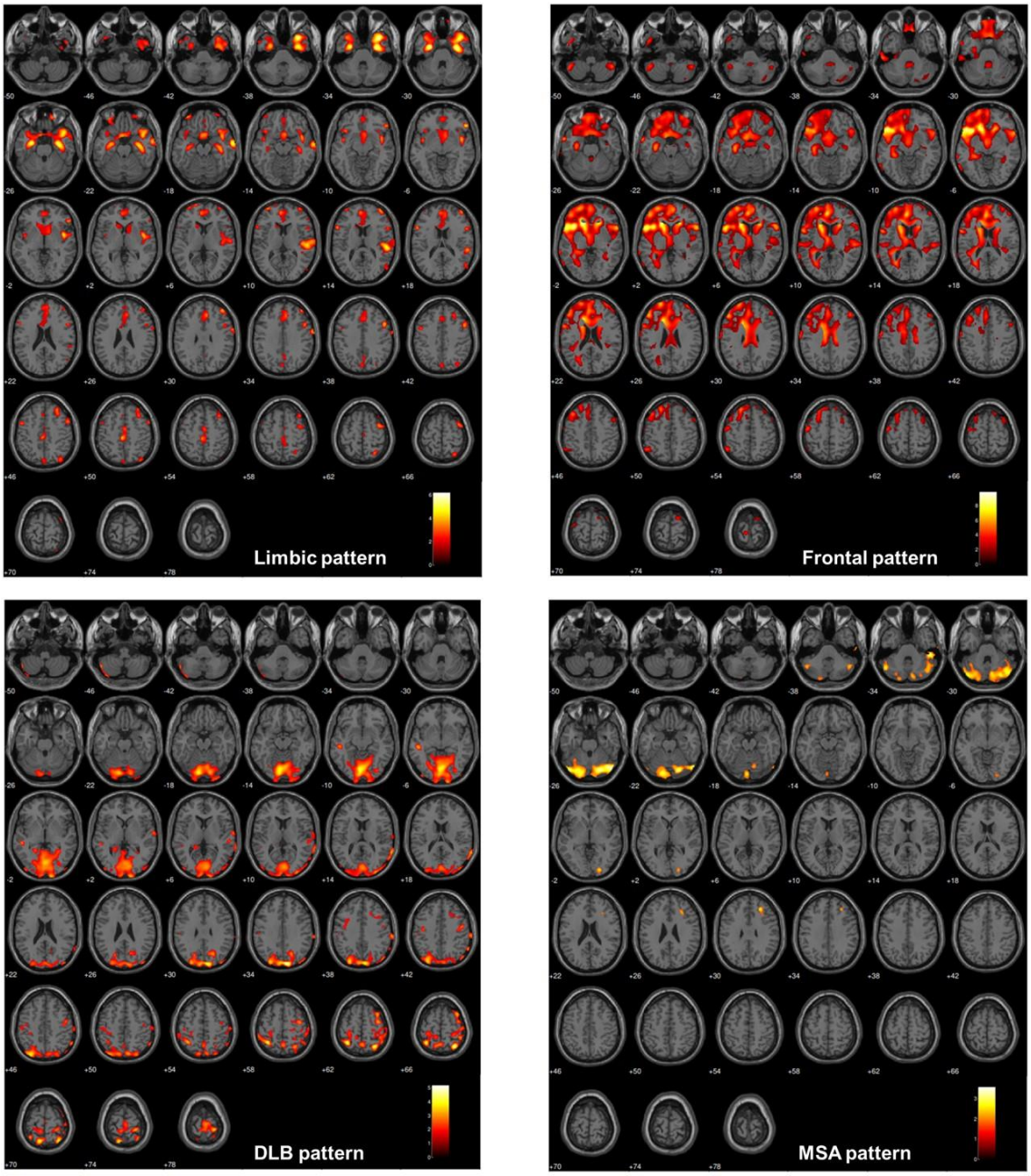


Figure 15. Examples of single-subject [^{18}F]FDG-PET SPM maps in Mild Cognitive Impairment individuals.

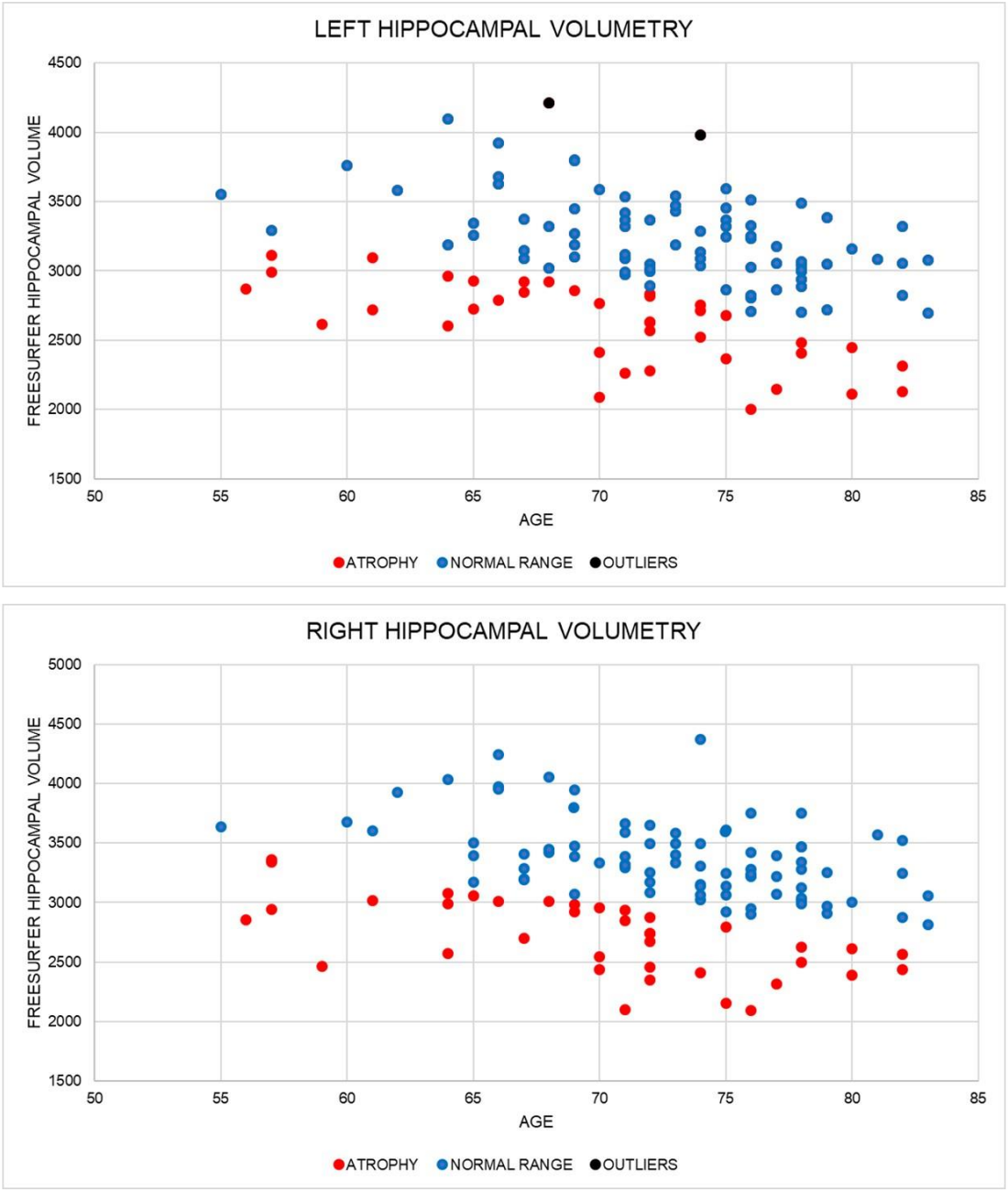


Figure 16. Hippocampal volume (left and right) in 120 MCI individuals with AD-like hypometabolism patter. Red circles indicate individuals whose hippocampal volume resulted below the fifth percentile in comparison with normal values, thus showing hippocampal atrophy.

Study 2. Biomarker-based stability in limbic-predominant amnestic mild cognitive impairment (Tondo et al., Eur J Neurol. 2020a)

MCI is considered an intermediate state between normal aging and dementia. This condition may be due to several possible etiologies, and it is very heterogeneous from the clinical and prognostic points of view. The amnestic type of MCI represents the most common prodromal stage of AD (Dubois *et al*, 2014). However, there is evidence of individuals with long-lasting memory deficits, signs of temporal lobe dysfunction, and long-term clinical stability (Cerami *et al*, 2018). The amnestic MCI subjects with focal temporal lobe dysfunction are usually aged individuals with isolated memory syndrome of the hippocampal type. In these limbic-predominant MCI cases, possible etiologies include hippocampal sclerosis, argyrophilic grain disease, PART, or the LATE, which has been proposed as the principal etiology in suspected non-AD pathology subjects and cognitive impaired individuals with evidence of neurodegeneration without concomitant tauopathy (Nelson *et al*, 2019). The limbic-predominant amnestic MCI subjects may be clinically indistinguishable from the amnestic MCI due to AD (Albert *et al*, 2011). Therefore, the investigation of biomarkers is crucial in this circumstance, especially considering that the selection of candidates for clinical trials in AD should avoid the recruitment of subjects clinically mimicking MCI due to AD, but who will remain stable over time (Iaccarino *et al*, 2019). *In vivo* biomarkers are decisive in MCI subjects for personalized medicine and in planning clinical trials, avoiding diagnostic and prognostic mistakes.

Several studies in the ADNI population suggested that the ATN system provides essential information in evaluating prognosis in amnestic MCI. Low CSF levels of amyloid-β or high cortical tracer uptake at the amyloid-PET represent valid proxies for amyloidosis in AD, while other biomarkers, including t-tau and p-tau CSF levels and brain atrophy as detected at the MRI, are less specific for AD (Jack Jr *et al*, 2020; Bittar *et al*, 2020). [^{18}F]FDG-PET, as a biomarker of neuronal dysfunction associated with neurodegenerative processes, can predict conversion from the MCI condition to AD dementia or other dementias with high accuracy, identifying brain hypometabolism patterns highly disease-specific already in the prodromal phase of dementia, and outperforming than other biomarkers including CSF and brain MRI (Caminiti *et al*, 2018;

Perani *et al*, 2016). In addition, [^{18}F]FDG-PET can predict long-term clinical stability, even in the presence of biomarker alterations suggestive for AD pathology (Iaccarino *et al*, 2019).

Data presented in this section are published in the study "Biomarker-based stability in limbic-predominant amnestic mild cognitive impairment" (Tondo *et al*, 2020a). The work aimed to define the role of *in vivo* biomarkers of neurodegeneration and pathology, including brain hypometabolism, as detected by [^{18}F]FDG-PET, and brain pathology, as studied by CSF analysis, in amnestic MCI individuals, by assessing the accuracy of each biomarker in estimating outcomes. Subjects were included from the HSR database and the ADNI database. A total of 142 amnestic MCI were involved, all having a long follow-up period (> 4 years), CSF analysis at the baseline, and [^{18}F]FDG-PET. According to the [^{18}F]FDG-PET SPM map at the baseline, amnestic MCI subjects were classified into two groups. The first group included limbic-predominant amnestic MCI, N = 80 (mean follow-up 8.2 ± 3.30 years), showing hypometabolism in the medial temporal lobe and limbic structures. The second group was composed of AD-like amnestic MCI, N = 62 (mean follow-up 6.47 ± 2.07 years), showing hypometabolism in the temporoparietal regions, precuneus, and posterior cingulate cortex. **Figure 17** shows results from the [^{18}F]FDG-PET SPM single-subject analysis, with examples of subjects showing the limbic-predominant hypometabolism pattern and prototypical subjects with the AD-like pattern. Cognitive, neuropsychological, and functional scores were available at the baseline and the follow-up for all included subjects. CSF measures included amyloid-β 42, t-tau, and p-tau levels, and they were evaluated both as a single measure and in combination (ratios), with the corresponding cutoff values provided by the manufacturer's recommendations and literature guidelines. amnestic MCI subjects in the two groups (limbic-predominant amnestic MCI and AD-like amnestic MCI) were further sub-classified according to the ATN system in subjects with an AD profile (A+, T+, N+ or A+, T-, N+) and subjects with a non-AD profile (A-, T+, N+ or A-, T-, N+), and progression to dementia was also compared according to the specific ATN profile. For a complete description of sample selection, clinical assessment, neuroimaging, CSF, and statistical analysis, see the "Materials and methods" section.

Table 1 lists cognitive, functional, CSF and genetic features in the limbic-predominant amnestic MCI and the AD-like amnestic MCI groups. At the baseline, all amnestic MCI

subjects had normal MMSE and CDR global scores, no impairment in daily functioning, no differences in cognitive and clinical assessments. No significant changes in clinical, cognitive, and functional measures were observed at the follow-up in the limbic-predominant amnestic MCI group. In contrast, the AD-like amnestic MCI group showed a significant drop in MMSE and CDR scores at the follow-up with significant concomitant impairment in daily functioning autonomy.

AMNESTIC MCI	LIMBIC-PREDOMINANT Baseline (N=80)	AD-LIKE Baseline (N=62)	*p-value*	LIMBIC-PREDOMINANT Follow-up (N=80)	AD-LIKE Follow-up (N=62)	*p-value*
Female/male	32/48	35/27	-	-	-	-
Education, years	13.69 ± 4.56	13.95 ± 4.57	0.733	-	-	-
Age, years	74.28 ± 5.40	71.14 ± 6.39	0.002	78.78 ± 5.26	73.76 ± 6.87	**0.000**
Disease duration, years	4.05 ± 2.44	4.05 ± 2.51	0.557	8.20 ± 3.30	6.47 ± 2.70	**0.000**
MMSE score	25.73 ± 2.06	25.40 ± 1.81	0.481	25.05 ± 2.58	19.67 ± 5.71	**0.000**
CDR score	0.50 ± 0.00	0.50 ± 0.00	-	0.56 ± 0.34	0.70 ± 0.25	**0.000**
IADL score	6.77 ± 1.31	7.01 ± 1.55	0.199	6.33 ± 1.13	5.45 ± 1.99	0.111
FAQ score	4.14 ± 5.98	4.14 ± 4.03	0.239	6.85 ± 8.83	9.98 ± 6.69	**0.009**
CSF amyloid-β positivity, N (%)	43 (54)	55 (89)	-	-	-	-
CSF t-tau positivity, N (%)	35 (44)	40 (65)	-	-	-	-
CSF p-tau positivity, N (%)	51 (64)	58 (94)	-	-	-	-
t-tau/amyloid-β pathological ratio	56 (70)	56 (90)	-	-	-	-
p-tau/amyloid-β pathological ratio	63 (79)	62 (100)	-	-	-	-
APOE ε 23 %	10	2	-	-	-	-
APOE ε 33 %	44	38	-	-	-	-
APOE ε 34 %	34	48	-	-	-	-
APOE ε 44 %	12	12	-	-	-	-

Abbreviations: AD, Alzheimer's disease; aMCI, amnestic mild cognitive impairment; APOE, apolipoprotein E; CDR, Cognitive Dementia Rating scale; CSF, cerebrospinal fluid; FAQ, Functional Activities Questionnaire; IADL, instrumental activities of daily living; MMSE, Mini-Mental State Examination; n, number of subjects; p-tau, phosphorylated tau; SD, standard deviation; t-tau, total tau

Table 1. Demographics, cognitive, functional, CSF and genetic features in the limbic-predominant amnestic MCI and the AD-like amnestic MCI groups (adapted from: Tondo *et al.*, 2020a).

Figure 18 shows the comparison between the two groups in cognitive and functional abilities scores, significantly impaired in the AD-like amnestic MCI group compared with the limbic-predominant amnestic MCI group. To estimate the cognitive decline's annual trajectory, we calculated the index of progression, indicating the number of MMSE points lost per year and based on the following formula: (MMSE score at follow-up – MMSE score at baseline) / years of follow-up. As underlined by Index of Progression, limbic-predominant amnestic MCI was associated with minimal or no changes in global cognitive status over a long follow-up (MMSE points lost per year −0.20 ± 0.70), in contrast to AD-like amnestic MCI, whose cognitive decline was significantly higher (MMSE points lost per year −1.50 ± 1.43).

At the follow-up, the conversion rate to dementia was 7% in the limbic-predominant amnestic MCI group and 86% in the AD-like group. [^{18}F]FDG-PET SPM map

classification predicted progression to dementia/stability with an overall accuracy of 90%. None of the considered clinical, cognitive, genetic variables, including age, sex, educational level, *APOE*, MMSE at the baseline, disease duration, predicted stability, or conversion to dementia. **Figure 19** represents survival curves indicating the probability of clinical stability in subjects stratified according to [^{18}F]FDG-PET patterns, and the accuracy of [^{18}F]FDG-PET single-subjects maps in predicting conversion with the receiver-operating characteristic curve analysis. According to the ATN classification, the limbic-predominant amnestic MCI group showed high variability in biomarker alterations, with the group approximately divided in half in the AD spectrum and a half in the non-AD spectrum. Conversely, about 90% of subjects in the AD-like group were classified as having an AD profile. While ATN classification did not help predict conversion/stability in the limbic-predominant group, the AD profile was strongly associated with conversion to dementia in the AD-like group, as expected. Most importantly, in the limbic-predominant amnestic MCI group, none of the CSF measures, alone or in combination, predicted stability or conversion to dementia. In addition, no differences were found in progression between the two subgroups with a different likelihood of AD pathology. In other words, subjects with limbic-predominant amnestic MCI did not progress to dementia regardless of the evidence of biomarker alterations suggestive for AD pathology.

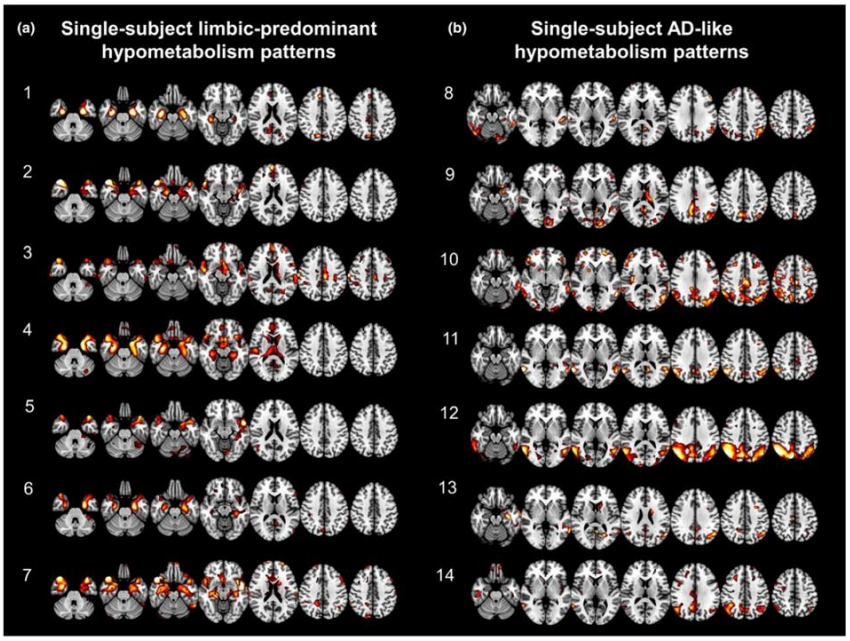


Figure 17. Examples of single-subject [^{18}F]FDG-PET hypometabolic patterns (source: Tondo *et al.*, 2020a)**.**

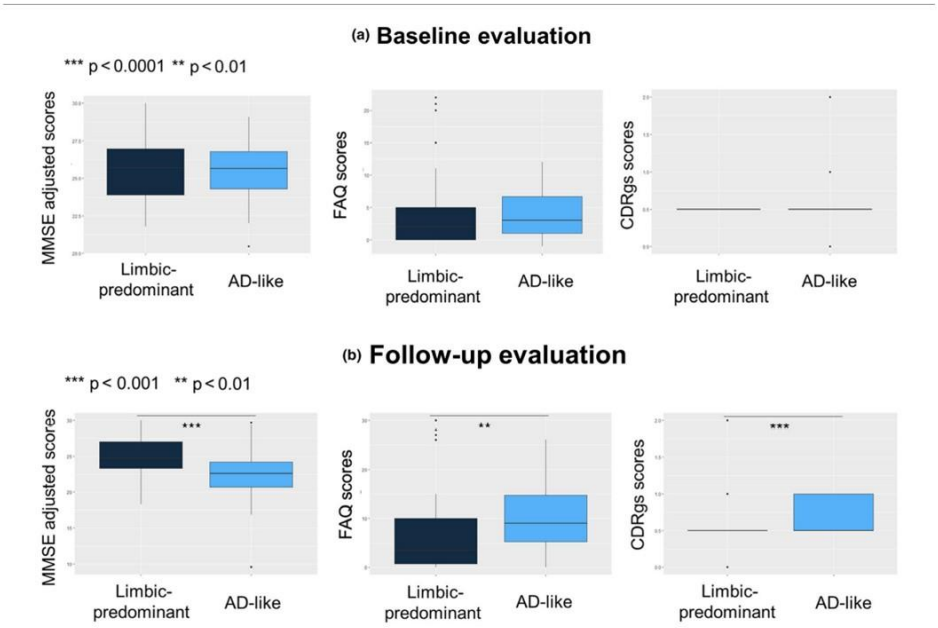


Figure 18. Baseline and follow-up cognitive and functional comparison between the limbic-predominant group and the AD-like group (source: Tondo *et al.*, 2020a).

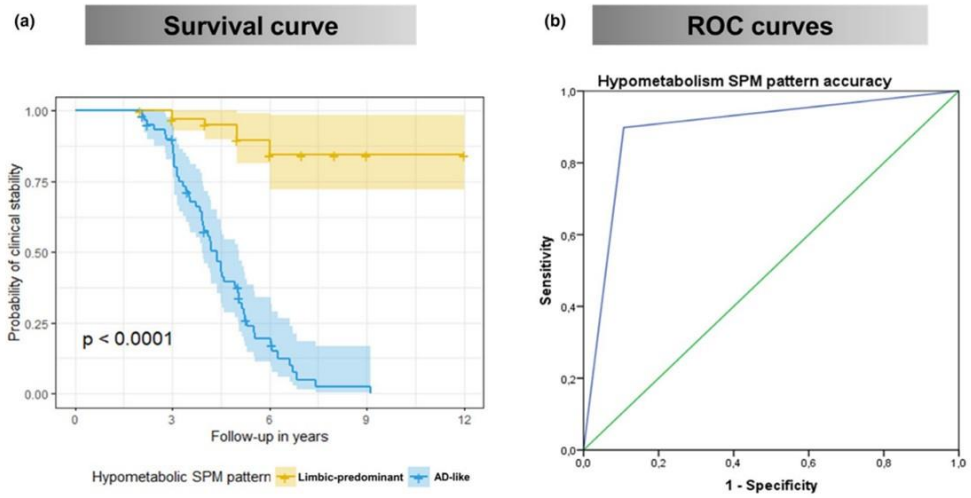


Figure 19. Survival curves indicating the probability of clinical stability in subjects stratified according to [^{18}F]FDG-PET patterns, and Receiving Operating Curves indicating the accuracy of [^{18}F]FDG-PET single-subjects maps in predicting conversion (source: Tondo *et al.*, 2020a).

Study 3. Brain metabolism and amyloid load in individuals with subjective cognitive decline or pre-Mild Cognitive Impairment

Neuropathological changes leading to dementia start years before symptoms onset, and the identification of people at risk for developing cognitive impairment is crucial for elaborating effective disease-modifying therapies. However, caution is needed when considering people in the preclinical dementia phase of dementia. SCD defines a self-experienced decline in cognitive functions with performance within the normal range on standardized cognitive assessment (Jessen *et al*, 2014). Individuals with pre-MCI are considered between normal cognition and the MCI stage, experimenting with cognitive decline from a previous level of function, even though current objective performance has not yet fallen below a cut-point for MCI based on group norms (Storandt *et al*, 2006). Biomarker information plays a central role in defining a risk profile in these conditions. According to recent criteria, the presence of subjective cognitive complaints or subtle cognitive decline, associated with the positivity of physio-pathological biomarkers, lead to a diagnosis of preclinical AD (Jack Jr *et al*, 2018a). Nevertheless, there is considerable evidence that only a minority of subjects fulfilling these criteria progress to dementia during follow-up (Dubois *et al*, 2021). The assessment of biomarkers of neurodegeneration and pathology in the subjective cognitive complaints and pre-MCI categories combined with long follow-up can be expected to provide useful information about the risk profile by identifying subjects who are not on a trajectory to dementia, avoiding exposure to possible side effects of the tested treatment.

This study aimed to investigate neurodegeneration and pathology in individuals with SCD and pre-MCI, searching for neuropsychological and neuropsychiatric correlations identifying distinct profiles already in the preclinical dementia phase. Included data derived from the clinical, prospective multi-center Network-AD project (AD-NET-02346784). The project involved N = 459 individuals in the dementia continuum, from the SCD stage to the overt dementia phase, to develop and validate operational research diagnostic criteria, integrating clinical, neuropsychological, CSF, and imaging measures, for early AD diagnosis in Memory Clinics. In the current study, individuals with SCD or pre-MCI were included, as defined by clinical criteria (Jessen *et al*, 2014; Duara *et al*, 2011). Subjects underwent baseline neuropsychological and neuropsychiatric evaluation

and baseline [^{18}F]FDG-PET. A subgroup of subjects also underwent amyloid-PET (see the "Materials and methods" section for a detailed description of sample characteristics, neuropsychological evaluation, imaging analyses, and statistical correlations). The total sample included N = 105 participants (SCD = 49; pre-MCI = 56). The cognitive and neuropsychological evaluation included the MMSE and tests assessing memory, language, attention, executive function, and visuospatial and constructive abilities. The neuropsychiatric evaluation included the Neuropsychiatric Inventory and the Starkstein Apathy Scale.

Table 2 lists demographics, clinical and neuropsychological features in the whole group and SCD and pre-MCI groups. The statistical comparison revealed significant differences between SCD and pre-MCI groups regarding neuropsychological evaluation, showing worse performances in the pre-MCI group, as expected. Neuropsychiatric symptoms were frequently reported in the whole sample, with 78% (N = 82) showing at least one symptom (defined as Neuropsychiatric Inventory total score ≥ 1). Regarding the neuropsychiatric sub-syndromes, the affective one was the most frequent (68%). **Table 3** reports the prevalence and severity of neuropsychiatric subsyndromes in the whole sample and in each group.

	Whole Sample	SCD	pre-MCI	*p-value*
DEMOGRAPHICS AND CLINICAL FEATURES				
N (%)	105	49 (46.7%)	56 (53.3%)	-
Age (mean±SD)	67.676±6.99	67.061±6.87	68.214±7.12	*p*=0.402
Sex female (%)	62 (59%)	33 (31%)	29 (27%)	-
MMSE (mean±SD)	28.857±1.376	29.280±1.085	28.495±1.501	***p*=0.003**
NEUROPSYCHOLOGICAL/NEUROPSYCHIATRIC EVALUATION				
NPI (frequency x severity)	5.923±5.745	5.755±5.797	6.071±5.748	*p*=0.880
NPI (caregiver distress)	3.009±4.005	2.755±3.585	3.232±4.360	*p*=0.948
Starkstein Apathy Scale	7.798±5.537	7.648±6.628	7.928±4.426	*p*=0.364
FCSRT immediate free recall	28.425±5.466	29.259±6.911	27.696±3.695	***p*=0.018**
FCSRT delayed-free recall	10.547±1.332	10.883±1.120	10.266±1.437	***p*=0.020**
Phonemic Fluency	37.415±12.188	38.153±12.351	36.769±12.117	*p*=0.345
Semantic Fluency	45.341±10.321	48.4±9.235	42.672±10.55	***p*=0.004**
Rey-Osterrieth Figure copy	34.11±2.202	34.224±1.877	34.013±2.460	***p*=0.629**
Rey-Osterrieth Figure recall	20.966±6.591	23.88±5.754	18.468±6.269	***p*=0.000**
Trail Making Test (A)	45.634±16.525	40.833±13.324	49.75±17.954	***p*=0.006**
Trail Making Test (B)	92.519±56.484	68.458±35.806	113.142±62.77	***p*=0.000**
Trail Making Test (B-A)	46.135±48.261	26.041±29.391	63.672±54.548	***p*=0.000**
CUBE-copying drawing	14.241±4.781	14.306±3.282	14.192±5.705	*p*=0.859

Abbreviations: F: female; FCSRT: Free and Cued Selective Reminding Test; M: male; MMSE: Mini Mental State examination; N: number; NPI: Neuropsychiatric Inventory; pre-MCI: pre Mild Cognitive Impairment; SCD: subjective cognitive decline; SD: standard deviation

Table 2. Demographics, clinical and neuropsychological features in the preclinical dementia groups.

	Whole Sample N = 105				SCD N = 49				Pre-MCI N = 56			
Syndromes	N	%	Score mean±SD	range	N	%	Score mean±SD	range	N	%	Score mean±SD	range
apathetic	25	24%	3.79±2.57	1-9	12	24%	3.83±2.48	1-9	13	23%	3.54±2.75	1-8
hyperactive	43	41%	3.83±3.93	1-20	17	35%	3.18±4.43	1-20	26	46%	4.46±3.54	1-14
affective	70	68%	4.41±2.27	1-12	36	73%	4.14±2.75	1-12	34	60%	4.32±2.09	1-9
psychotic	20	19%	3.05±1.17	1-6	10	20%	3.30±1.16	2-6	10	20%	3.10±1. 52	1-6
at least 1 psychiatric symptom	82	78%	7.46±5.41	1-29	40	81%	7.05±5.75	2-29	42	75%	8.09±5.24	1-25

Abbreviations: N, number; SD, standard deviation; pre-MCI, pre-mild cognitive impairment; SCD, subjective cognitive decline

Table 3. Prevalence and severity of neuropsychiatric subsyndromes in the preclinical dementia groups.

[^{18}F]FDG-PET images were analyzed using the single-subject SPM standardized procedure (Perani *et al*, 2014a; Della Rosa *et al*, 2014). SPM analysis revealed the absence of metabolism abnormalities in 45% of subjects, while 55% showed brain hypometabolism. Three neuroimaging experts classified Single-subject brain hypometabolism SPM maps, showing near-perfect agreement ('Cohen's kappa'> 0.95). The visual rating of SPM maps allowed to classify subjects into one of five patterns (for a complete description, see Study 1): a) normal brain metabolism; b) AD-like pattern; c) DLB-like pattern; d) frontal-like pattern; e) limbic-predominant (medial-temporal) pattern. b) temporoparietal hypometabolism, namely Alzheimer's disease (AD)-like pattern. **Table 4** shows the rate of hypometabolism patterns in the whole sample and separately in the SCD and pre-MCI subgroups. **Figure 20** presents single-subject SPM map explicative cases. Analyzing neuropsychological performances in each metabolism pattern group, some significant differences emerged. The limbic-predominant group showed lower scores at the semantic fluency test than normal brain metabolism group (p=0.044). The frontal-like group showed higher Neuropsychiatric Inventory scores (both frequency x severity total scores and caregiver distress scores) than the normal brain metabolism group (p = 0.008), meaning a higher rate of neuropsychiatric disturbances in the frontal-like hypometabolism group.

FDG-PET SPM pattern	Whole Sample N = 105	%	SCD N = 49	%	Pre-MCI N =56	%
Normal Scan	47	45%	28	56%	19	34%
AD-like	11	11%	4	8%	7	13%
DLB-like	10	9%	3	6%	7	11%
Frontal-like	30	28%	12	23%	18	30%
Limbic-predominant	7	7%	2	4%	5	9%

Abbreviations: SCD, subjective cognitive decline; pre-MCI, pre-mild cognitive impairment; AD, Alzheimer's disease; DLB, Dementia with Lewy bodies; N, number

Table 4. Rate of different hypometabolism patterns in the preclinical dementia groups.

Amyloid-PET was available for N = 60 subjects, and it was analyzed following a validated method to obtain cortical global and regional Standard Uptake Value ratio (SUVr). Based on the literature recommendation, the quantification method allowed dichotomous classification in amyloid positive and amyloid negative subjects (Ong *et al*, 2015). Considering the selected cutoff, 18% (N = 11) of 60 subjects were classified as amyloid positive, and 82% (N = 49) as amyloid negative. Seven amyloid positive subjects also had an abnormal [^{18}F]FDG-PET hypometabolism pattern (three presented an AD-like hypometabolism pattern, while four showed a frontal-like pattern). Four subjects showed abnormal cortical amyloid load and no signs of neurodegeneration at the [^{18}F]FDG-PET scan. In the case of amyloid negative subjects, 18/49 had a normal [^{18}F]FDG-PET scan, while 31/49 showed an altered [^{18}F]FDG-PET hypometabolism.

Considering the availability of neuropsychological, neuropsychiatric, and metabolism data, we tested whether distinct profiles with different metabolism correlates exist in the preclinical dementia phase. Thus, principal component analysis was employed to explore neuropsychological and neuropsychiatric profiles. Specifically, two principal component analyses were applied in the whole sample and correlated with brain metabolism data. In the neuropsychological principal component analysis, three components emerged: 1) executive/visuomotor 2) memory, and 3) visuospatial/constructional. In the neuropsychiatric principal component analysis emerged two components: 1) affective and 2) hyperactive/psychotic. **Table 5** lists the variables included in each component. Significant correlations between brain metabolism and the principal component analysis factors emerged. **Figure 21** represent these correlations. The executive/visuomotor factor showed a significant negative correlation with metabolism (i.e., linear decrease in glucose metabolism together with impaired performance, thus higher scores) in the superior and the middle frontal gyri, lingual gyrus, cuneus, precuneus, and middle cingulate cortex, plus the caudate nuclei and thalamus, bilaterally. The memory factor showed a significant positive correlation with metabolism (i.e., linear decrease in glucose metabolism and decreasing scores) in the precuneus, cuneus, superior and inferior parietal lobules, the posterior and middle cingulate cortices, the superior and the middle frontal gyri. The visuospatial/constructional factor showed a significant positive correlation with right-lateralized metabolism (i.e., linear decrease in glucose metabolism and decreasing scores of grouped tests), specifically in the angular gyrus, the anterior and middle cingulate

cortex, the dorsolateral frontal cortex. The affective factor showed a significant negative correlation with brain metabolism (i.e., linear decrease in glucose metabolism together with increasing symptoms) mainly in the right hemisphere, in the insula, temporal pole, the anterior cingulate cortex, superior temporal gyrus, and in the inferior frontal gyrus (pars orbitalis). The hyperactive/psychotic factor showed a significant negative correlation with metabolism (i.e., linear decrease in glucose metabolism together with increasing symptoms) in the orbitofrontal cortex and prefrontal cortex, the anterior cingulate cortex, the left lateral temporal cortex, insula, and the caudate. Lastly, the voxel-wise multivariate regression models were run separately for pre-MCI and SCD subgroups (**Figure 21**). The executive/visuomotor factor negatively correlated with metabolism in extended temporoparietal and occipital regions in the pre-MCI group, whereas in the SCD group, this factor correlated mainly with subcortical structures. The pre-MCI group trained results regarding the memory factor, showing positive correlations between memory and metabolism in the frontal-temporal-parietal cortex. Correlations in the visuospatial/constructional factor were more represented in the right hemisphere and broadly similar in the two groups. Both groups' affective factors correlated with insular and temporal medial metabolism, whereas the hyperactive/psychotic factor correlated more extensively with frontal, temporal, and insular regions in the SCD group.

A Neuropsychological assessment	1– Executive /visuo-motor	2– Mnemonic	3– Visuo-spatial /constructional
FCSRT immediate free recall	-0,106	**0,872**	-0,002
FCSRT delayed-free recall	-0,079	**0,813**	-0,101
Phonemic Fluency	-0,095	0,471	0,303
Semantic Fluency	-0,377	**0,676**	0,071
Rey-Osterrieth Figure copy	0,054	0,057	**0,824**
Rey-Osterrieth Figure recall	-0,185	0,387	0,412
Trail Making Test (A)	**0,776**	-0,186	0,160
Trail Making Test (B)	**0,909**	-0,182	-0,299
Trail Making Test (B-A)	**0,790**	-0,169	-0,407
CUBE- copying drawing	-0,223	-0,068	**0,610**
B Neuropsychiatric assessment	**1 – Affective**	**2 – Hyperactive/psychotic**	
Starkstein Apathy Scale	**0,734**	0,043	
NPI apathetic	**0,708**	0,103	
NPI hyperactivity	0,147	**0,739**	
NPI affective	**0,676**	0,074	
NPI psychotic	0,016	**0,806**	

Abbreviations: FCSRT: Free and Cued Selective Reminding Test; NPI: Neuropsychiatric Inventory

Table 5. Neuropsychological and neuropsychiatric factors emerging from the principal component analyses.

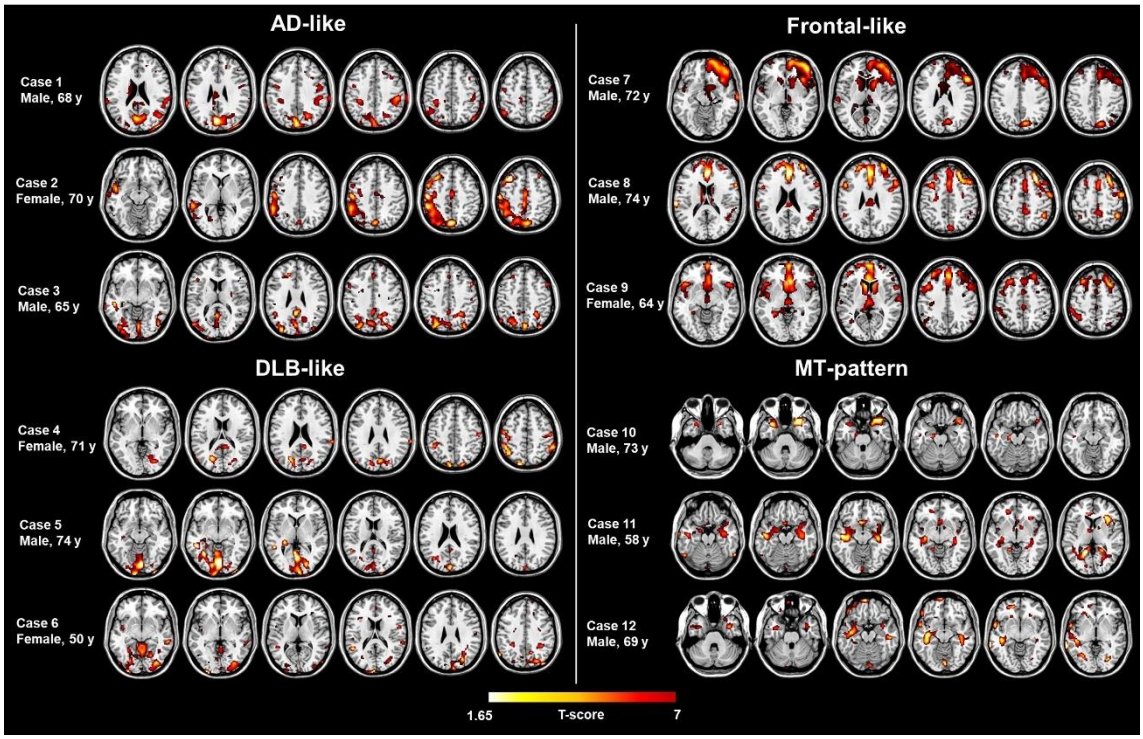


Figure 20. Single-subject explicative cases of hypometabolism patterns in the preclinical dementia groups.

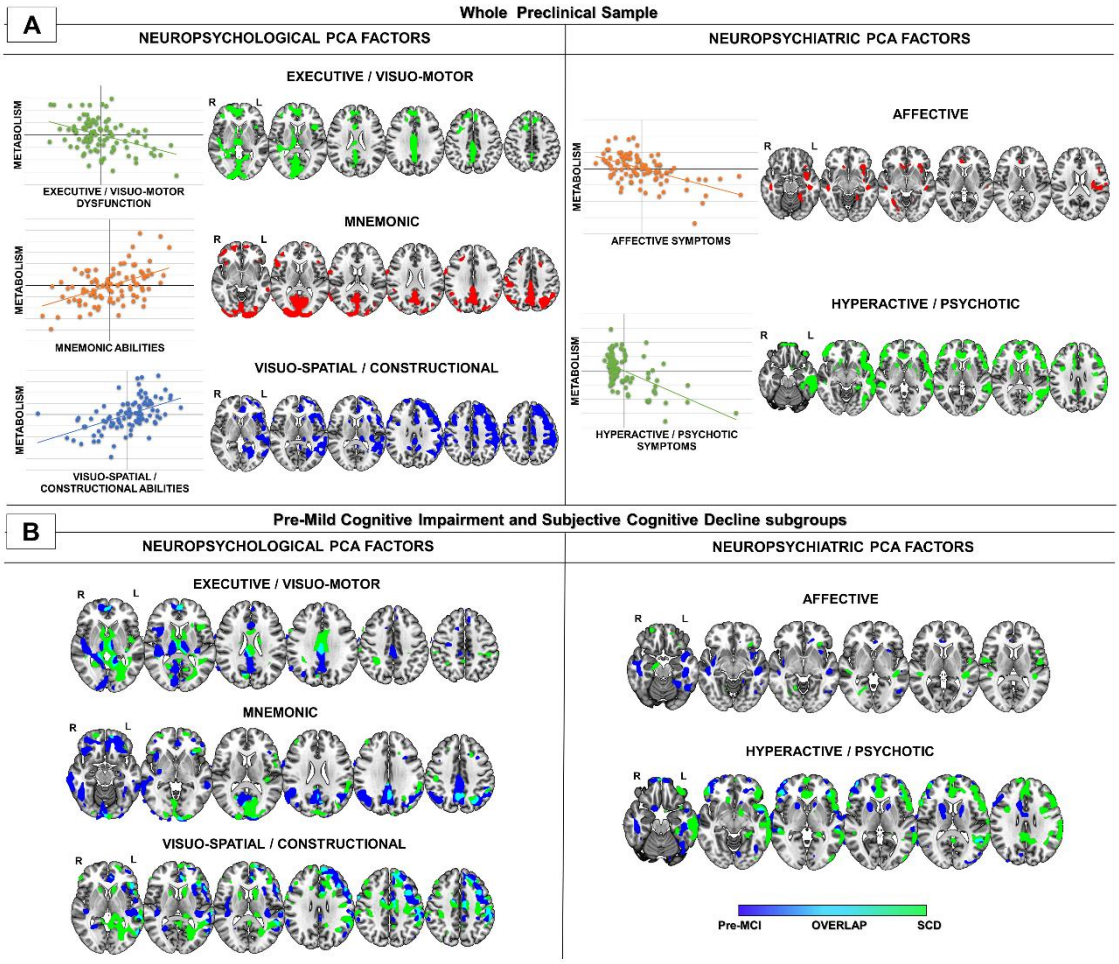


Figure 21. Neuropsychological/Neuropsychiatric and metabolism correlations in the different factors emerging from the principal component analysis, in the whole preclinical dementia group and separately for each subgroup.

Part 2. PET imaging evaluating the relationship between neuroinflammation and neurodegeneration in neurodegenerative diseases

Review 3. TAM Receptor Pathways at the Crossroads of Neuroinflammation and Neurodegeneration (Tondo et al., Dis Markers. 2019)

Neurodegenerative diseases represent the leading cause of morbidity and mortality worldwide, and their global socioeconomic and public health impact is enormous, progressively intensified by the aging community (Cova *et al*, 2017). Despite specific pathological characteristics, neurodegenerative processes in different disorders are frequently associated with chronic dysregulation of neuroinflammatory responses. The complete understanding of neuroimmune mechanisms participating in neurodegeneration may be the key to modulate neuroinflammatory pathways, possibly resulting in new experimental therapies. The first evidence on the clinical benefit of modulatory actions on neuroinflammatory responses came out from studies showing a negative correlation between the use of nonsteroidal anti-inflammatory drugs and the likelihood of developing AD (In'T Veld *et al*, 2001). However, the broad suppression of neuroinflammation seems to be ineffective in improving the disease progression. Clinical trials using anti-inflammatory drugs, including steroids, nonsteroidal anti-inflammatory drugs, and selective inhibitors of cyclooxygenase-2, failed to demonstrate effectiveness in AD (Jaturapatporn *et al*, 2012). This aspect underlines the challenge of obtaining clinically relevant effects without producing remarkable adverse reactions by modulating inflammatory responses, implying both detrimental and beneficial mechanisms (Cappellano *et al*, 2013). In addition, the modulation of adaptive immunity, both with active or passive immunization therapies, has failed to meet the expectation due to not negligible immunological side effects, including the appearance of microhemorrhage and occurrence of vasogenic edema (Morgan, 2011). Lastly, the problem of the right target should be considered, namely the inclusion in clinical trials of patients whose neurodegenerative condition is not yet irreversible. The potential of therapies targeting neuroinflammation is greatly reduced when neurodegeneration has already started. Thus, they should be tested ideally in a pre-symptomatic period (Imbimbo, 2009).

Tyro3, Axl, and Mertk (TAM receptors) are tyrosine kinase receptors involved in multiple roles in CNS and peripheral nervous system, including neuroinflammatory responses. The interest in molecular pathways implying TAMs activation arises from the evidence of their involvement in neurodegenerative diseases, including AD, PD, and ALS. The following discussion is based on the published review "TAM Receptor Pathways at the Crossroads of Neuroinflammation and Neurodegeneration" (Tondo *et al*, 2019), providing an overview of the role of TAMs in neurodegenerative diseases, underlying the therapeutic potential of their modulation. TAMs are cell surface receptors transmitting signals from the extracellular space to the cell cytoplasm and nucleus. The most studied TAMs ligands are the Growth Arrest Specific 6 (Gas6) and Protein S. Both TAMs and their ligands are widely expressed in multiple organs and tissues, including the CNS and peripheral nervous system, and are involved in multiple functions (Pierce & Keating, 2014). TAMs are modulators of neurogenesis and regulate neural stem cells survival and differentiation (Ji *et al*, 2014). In CNS, they are involved in synaptogenesis and regulation of synaptic plasticity (Prieto *et al*, 2007). TAMs stimulate peripheral nerve repair and myelination and modulate cell proliferation (Wium *et al*, 2018). As a consequence, they are involved in cancer genesis. For instance, Gas6 and Axl are overexpressed in the human glioblastoma multiforme, associated with a poor prognosis (Hutterer *et al*, 2008), and schwannoma development (Ammoun *et al*, 2014).

TAMs finely regulated inflammatory responses. In particular, the modulation of microglia activation is of high interest. TAMs regulate microglial phagocytic activity and the clearance of cell waste products (Fourgeaud *et al*, 2016). Specifically, Axl activities are predominant in pro-inflammatory responses, and Axl deficits are related to delayed phagocytosis and prolonged induced axonal damage (Hoehn *et al*, 2008). Furthermore, the inefficient phagocytic activity affects myelination and remyelination after nerve damage due to the ineffective clearance of myelin debris (Binder & Kilpatrick, 2009). In both *in vivo* and *in vitro* studies, the loss of TAMs, specifically of Tyro3, which is highly expressed in oligodendrocytes, determines delayed myelination and reduced myelin thickness (Akkermann *et al*, 2017). In addition, Gas6, which promotes the survival of oligodendrocytes and Schwann cells, stimulates remyelination after neural damage (Lutz *et al*, 2017). Consequently, Gas6 knockout mice have remyelinating alterations linked to

microglia activation since the Gas6/Axl signaling has anti-inflammatory properties (Ray *et al*, 2017).

The TAMs activity has been related to several neurodegenerative processes, but evidence derives mainly from animal studies, and fewer data are reported in patients. By regulating the phagocytic activity of microglia cells, TAMs can be involved in pathological protein deposition. This aspect has been particularly studied in animal models of AD. In APP-mouse models, the overexpression of the receptor Tyro3 significantly reduces the amyloid-β deposition, while the absence of Tyro3 is associated with an increased rate of amyloid-β plaques hippocampal amyloid-β deposition (Zheng *et al*, 2012). In addition, stimulating the Axl-mediated pathway in APP/PS1 mouse model facilitates amyloid-β plaques clearance and improves cognitive impairment (Zhang *et al*, 2018). In humans, the examination of TAMs activity is less straightforward. CSF levels of Axl, together with other proteins such as chromogranin A and angiotensin-converting enzyme, correlated with longitudinal CSF reduction of amyloid-β42, thus with cortical amyloid deposition, in healthy people, suggesting that TAM modulation might be important in cognitively unimpaired individuals at risk for AD (Mattsson *et al*, 2013). Another study identified Axl and other twelve plasma markers, including complement C3, cortisol, IL-3, IL-13, matrix metalloproteinase-9 total, and APOE, whose levels correlated with amyloid-β cortical deposition, as measured by [^{11}C]PiB-PET, in AD patients from the ADNI database. Increased levels of Gas6 have been reported in CSF of AD patients compared with controls, suggesting a possible initial protective role since Gas6 can downregulate pro-inflammatory cytokines production and stimulate amyloid-β clearance (Sainaghi *et al*, 2017). Upregulation of genes related to Gas6 and the TLRs signaling have been reported from cognitively normal individuals to AD patients, again suggesting a dysregulation in the TAMs pathway as responsible for altered neuroinflammatory responses in AD pathogenesis (Herrera-Rivero *et al*, 2019). The link between TAMs dysregulation and neurodegeneration has been hypothesized in other neurodegenerative conditions. In a PD mouse model, the overexpression of α-synuclein was also associated with increased expression of Axl. In the same model, Axl and Tyro3 loss produced clinical improvement. In this case, exaggerated and deleterious response of microglia has been proposed, mediated by TAMs (Fourgeaud *et al*, 2016). In addition, the loss of the transcriptional factor Nrf2, which is involved in Axl and Mertk regulation

of microglia phagocytosis, exaggerate neuroinflammation, α-synuclein deposition, and neuronal loss in PD mice (Lastres-Becker *et al*, 2012). In human *post-mortem* transcriptomic analysis of ALS patient brains, a disruption in components of the epithelial layer of the choroid plexus has been shown. The choroid plexus is essential for BBB integrity, and its disruption favors leucocytes infiltration in the brain parenchyma. The leucocytes infiltrated in the ALS brain express pro-inflammatory cytokines and chemokines, such as the Macrophage Colony-Stimulating Factor and the Vascular endothelial growth factor A, favoring the infiltration of Mertk positive macrophage (Saul *et al*, 2020).

The interest in TAMs multiple activities relies on the fact that these receptors represent potential targets for experimental therapies. For instance, in an arthritis mouse model, Gas6 administration can obstacle inflammatory hyperactivation, producing clinical benefit (Van den Brand *et al*, 2013). However, the employment of TAMs modulation therapy in neurodegenerative disorders is challenging. The multiple TAMs mediated functions imply that therapies cannot simply block or enhance their activities. For example, Mertk prolonged blockage induces blindness in rats (Liu *et al*, 2015), and an indiscriminate TAMs inhibition induces altered autoimmunity responses (Xu *et al*, 2018). Conversely, promising results have been obtained regarding cancer therapy, where Axl inhibition in astrocytoma cells favors apoptosis and enhances sensitivity to chemotherapy (Keating *et al*, 2010). These data need *in vivo* confirmation but encourage the fine modulation of the molecular mechanisms underlying neuroinflammatory responses, which might provide clinical benefit.

Study 4. The combined effects of microglia activation and brain glucose hypometabolism in early-onset Alzheimer's disease (Tondo et al., Alzheimers Res Ther. 2020b)

EOAD is an AD subtype characterized by symptoms' onset before 65 years, greater disease severity, and faster disease progression than LOAD (Wattmo & Wallin, 2017). The onset in EOAD is often atypical, manifesting with problems in language, visuospatial and visuo-constructive abilities disturbances, or deficits in executive functions and behavioral abnormalities (Mendez, 2019). The more severe clinical picture in EOAD than in LOAD has been associated with more extensive pathology (Bigio *et al*, 2002), more significant synaptic dysfunction as detected by [^{18}F]FDG-PET (Kim *et al*, 2005), and greater neuronal loss as testified by brain atrophy (Frisoni *et al*, 2005). EOAD and LOAD patients also present distinct brain network organization features compared to healthy controls. EOAD shows more severe abnormalities in brain network connectivity at both global and regional levels, especially in the cingulate and occipital cortex (Chung *et al*, 2016). The occurrence of neuropsychiatric disturbances, frequent in EOAD, has been related to brain network connectivity involving frontal regions and limbic structures (Ballarini *et al*, 2016). PET imaging of neuroinflammation has been largely used in LOAD, but few data are available in EOAD (Kreisl *et al*, 2013, 2017). Using the second-generation TSPO tracer [^{11}C]PBR28, a study showed greater cortical microglia activation in EOAD than in LOAD patients. TSPO overexpression was particularly evident in the parietal cortex and the striatum, associated with worse neuropsychological performances and lower grey matter volume (Kreisl *et al*, 2013). In addition, EOAD patients showed a higher longitudinal increase of [^{11}C]PBR28 binding in parietal, occipital and frontal cortex than LOAD, again correlating with a faster clinical deterioration (Kreisl *et al*, 2017). These data suggest that microglia activation may be associated with clinical deterioration, reflecting neurodegeneration, along the disease course in EOAD.

The following data have been published in the study "The combined effects of microglia activation and brain glucose hypometabolism in early-onset Alzheimer's disease" (Tondo *et al*, 2020b). The study aimed to investigate the relationship between microglia activation and brain glucose metabolism in EOAD, using [^{11}C]PK11195-PET and [^{18}F]FDG-PET. Brain metabolic connectivity was also explored in the EOAD group

compared with normal controls to reveal possible long-distance network effects of microglia activation. The study included twelve AD patients whose symptoms' onset had occurred before 65 years. All patients underwent neuropsychological evaluation, brain MRI, [^{18}F]FDG-PET and [^{11}C]PK11195-PET, and lumbar puncture to collect CSF measures of amyloid-β, t-tau, and p-tau. Brain hypometabolism pattern was assessed at the group and the single-subject level, using the HSR database of N = 112 normal controls for SPM comparison. TSPO overexpression was derived at the group and at the single-subject level exploring voxel-wise statistical differences between the EOAD patients and nine healthy controls (mean age 43.6 ± 11.2 years). The area of interaction between the TSPO-PET signal and [^{18}F]FDG-PET hypometabolism map was calculated, and cerebral glucose metabolic rate and [^{11}C]PK11195-PET binding potentials were extracted from this region to verify the statistical correlation between the two markers. Using the interaction area as a seed, we performed a seed-based interregional correlation analysis to explore metabolic connectivity in the EOAD group compared to a group of 20 age and sex-matched healthy controls. See the "Materials and methods" section for further details on participants, imaging acquisition and analysis, statistical analysis.

The sample included four patients with typical AD, six with the frontal AD variant, and two PCA patients. The demographics and neuropsychological data of each patient are presented in **Table 6**. The single-subject [^{18}F]FDG-PET analysis revealed a consistent pattern of temporoparietal hypometabolism in all EOAD patients, with variable involvement of occipital and frontal regions according to the specific clinical phenotype, namely the posterior or the frontal AD variant. The single-subject [^{11}C]PK11195-PET analysis showed significant binding potentials increases in all EOAD patients in temporal, parietal, occipital, and frontal regions.

EOAD	01	02	03	04	05	06	07	08	09	10	11	12
Age, years	68	55	56	64	58	59	64	58	54	56	63	66
Education, years	5	13	13	8	13	8	8	8	8	13	8	5
Sex	f	m	m	f	f	m	f	f	m	f	f	m
Disease Duration, years	5	1	1	3	2	1	1	5	1	2	3	2
Diagnosis	tAD	tAD	tAD	tAD	fAD	fAD	fAD	fAD	fAD	fAD	PCA	PCA
MMSE	20	21	17	22	18	10	12	18	17	22	21	21
Token Test	31	31	23.5	29	23	11.5	19.25	22.25	33	31	27.5	30.5
PVF	27	10	7	29	16	6	3	13	22	31	56	44
SVF	30.1	8	18.5	42.8	12	16	8	13	45.5	44	29.2	59.2
Span Forward	6.39	4.84	2.83	5.13	5.75	6.13	2.92	5.04	2.92	5.83	4.13	6.02
Span Backward	4.53	< 3	< 3	3.19	< 3	< 3	< 3	3.1	< 3	3.79	4.19	< 3
RAVLT IR	22.1	14.8	13.8	24.3	10	UT	UT	0	36	41	21.5	32.3
RAVLT DR	0	0	0	0	1.5	UT	UT	0	4.2	4	3	3.6
ROF recall	8	UT	0	8.5	5.25	UT	0	5	5.75	11.75	0	7.5
ROF copy	9	UT	0	23	13.25	3	7.25	28.5	29.25	14.75	0	24.25
CDT	1	10	2	5	5	0	0	0	10	7	0	10
Attentive Matrix	36	UT	12.5	31.25	23	UT	17.5	23	22	49	34.25	23
Stroop time	33.5	28	44.25	36.75	29.25	UT	UT	UT	46.8	46.17	44.25	27
Stroop Errors	9.5	3.5	13	11.25	26.5	UT	UT	UT	5	0	5.25	2.5
Raven PM	19.5	UT	14	19	UT	UT	UT	21.5	26.5	22	12	31.5

Abbreviations: EOAD early-onset Alzheimer disease, tAD typical AD, fAD frontal Alzheimer disease, PCA posterior cortical atrophy, MMSE mini mental state examination, PVF phonemic verbal fluency, SVP semantic verbal fluency, RAVLT Rey auditory verbal learning task, IR immediate recall, DR delayed recall, ROF Rey-Osterrieth figure, CDT clock drawing test, Raven PM Raven Progressive Matrices, UT untestable

Table 6. Demographics and neuropsychological data of each early-onset Alzheimer's disease patient (adapted from: Tondo *et al.*, 2020b).

Figure 22 shows, for each subject, the hypometabolism and the [^{11}C]PK11195 binding potential maps. EOAD01 is a typical AD patient with moderate cognitive impairment characterized by memory deficits, visuospatial difficulties, and mood disturbances. [^{18}F]FDG-PET SPM map shows marked hypometabolism in the temporoparietal cortices, mainly in the right hemisphere. [^{11}C]PK11195 binding potentials are increased in temporoparietal regions, predominantly on the right and occipital regions. In addition, the topographical overlap between TSPO overexpression and brain hypometabolism is present in temporoparietal regions. EOAD02 is a typical AD patient with moderate cognitive impairment characterized by memory deficits and minor language and executive functions deficits. [^{18}F]FDG-PET SPM map reveals hypometabolism in temporoparietal cortices. [^{11}C]PK11195-PET shows increased binding potentials in temporoparietal regions and occipital and frontal regions. Topographical overlap between TSPO overexpression and brain hypometabolism is evident in temporoparietal regions. EOAD03 is a typical AD patient with severe cognitive impairment and diffused deficits in memory, executive, language, and visuospatial domains. [^{18}F]FDG-PET shows hypometabolism in temporoparietal cortices. [^{11}C]PK11195-PET shows increased binding potentials in temporoparietal regions, in posterior cingulate and occipital cortices. Temporo-parietal regions represent the regions of spatial overlap between TSPO

overexpression and brain hypometabolism. EOAD04 is a typical AD patient with moderate cognitive impairment characterized by memory, executive, and visuospatial deficits. [^{18}F]FDG-PET underlines hypometabolism in temporoparietal regions, specifically the middle temporal gyrus and the bilaterally angular gyrus. [^{11}C]PK11195-PET shows increased binding potentials in temporal and occipital regions, partly involving parietal and frontal cortices and subcortical structures. Temporo-parietal regions are the regions of spatial overlap between TSPO overexpression and brain hypometabolism. EOAD05 is a frontal AD variant patient with moderate-severe cognitive impairment characterized by executive and memory deficits, with minor language and visuospatial disturbances associated with behavioral and mood disorders. [^{18}F]FDG-PET SPM map shows marked hypometabolism in the temporoparietal and frontal regions. [^{11}C]PK11195-PET shows increased binding potentials in frontal, temporal-parietal, and occipital regions, with subcortical distribution. Topographical overlap between TSPO overexpression and brain hypometabolism is present in temporoparietal and frontal regions, mainly in the right hemisphere. EOAD06 is a frontal AD variant patient with very severe cognitive impairment and marked behavioral disturbances. [^{18}F]FDG-PET shows extensive hypometabolism in frontal and temporoparietal cortices, with right side prevalence. [^{11}C]PK11195-PET shows diffused increased binding potentials in frontal, temporal, parietal, and occipital cortices. Temporal, parietal and frontal regions are areas of overlap between TSPO overexpression and brain hypometabolism. EOAD07 is a frontal AD variant patient with severe cognitive impairment characterized by diffused memory, executive, language, visuospatial deficits, behavioral disturbances, and hallucinations. [^{18}F]FDG-PET SPM map reveals hypometabolism in the prefrontal cortex and temporoparietal regions bilaterally. [^{11}C]PK11195-PET shows increased binding potentials in temporoparietal regions, precuneus, and occipital and frontal lobes. Topographical overlap between TSPO overexpression and brain hypometabolism is evident in temporoparietal and frontal regions. EOAD08 is a frontal AD variant patient with moderate cognitive impairment characterized by language, executive, visuospatial and memory deficits. [^{18}F]FDG-PET SPM map shows hypometabolism in frontal and temporoparietal regions, namely the inferior and middle frontal gyrus on the left and bilaterally the middle temporal gyrus inferior parietal lobule, precuneus, and posterior cingulate cortex. [^{11}C]PK11195 binding potentials are increased in temporal, occipital,

and frontal regions. Topographical overlap between TSPO overexpression and brain hypometabolism is present in temporal and frontal regions. EOAD09 is a frontal AD variant patient with severe cognitive impairment and marked behavioral disturbances, including sweet craving, irritability, and sexual disinhibition. [^{18}F]FDG-PET SPM map shows extensive hypometabolism in the right orbitofrontal and dorsolateral cortex, temporal cortex, angular gyrus, precuneus, and posterior cingulate cortex. [^{11}C]PK11195-PET shows increased binding potentials in the orbitofrontal cortex and subcortical structures. TSPO overexpression and brain hypometabolism overlapped in frontal and temporoparietal regions. EOAD10 is a frontal AD variant patient with moderate cognitive impairment characterized by executive, visuospatial and behavioral disturbances. [^{18}F]FDG-PET SPM map reveals hypometabolism in frontal and temporoparietal cortices, especially in the right hemisphere. [^{11}C]PK11195 binding potentials are increased in temporal and occipital regions. Thus, the overlap between TSPO overexpression and brain hypometabolism involves temporal regions mainly in the right hemisphere. EOAD11 is a PCA patient with moderate cognitive impairment characterized by memory, executive, and visuospatial disturbances. [^{18}F]FDG-PET SPM map highlights marked hypometabolism in occipital and temporal cortices bilaterally. [^{11}C]PK11195 binding potentials are increased in occipital and temporal regions. Therefore, the overlap between TSPO overexpression and brain hypometabolism involves temporal and occipital regions. EOAD12 is a PCA patient with moderate cognitive impairment characterized by visuospatial and memory deficits. [^{18}F]FDG-PET SPM map shows hypometabolism in occipital and temporoparietal cortices. [^{11}C]PK11195-PET shows increased binding potentials in occipital temporal and parietal regions. Hypometabolism overlapped with TSPO overexpression in temporal, parietal, and occipital areas.

The group-level study confirmed the commonalities in the topographical distribution of hypometabolism and microglia activation. The group-level [^{18}F]FDG-PET analysis showed the typical AD hypometabolism pattern involving temporoparietal regions and, to a lesser extent, occipital and frontal regions. The high rate in the sample of atypical AD patients likely drove the presence of significant frontal and occipital hypometabolism. The group-level [^{11}C]PK11195-PET analysis showed higher TSPO expression in EOAD patients than controls in temporoparietal cortices and, in smaller measures, in occipital

and frontal regions. A region of spatial overlap between microglia activation and brain hypometabolism in the right temporoparietal junction emerged from the interaction analysis. Specifically, the interaction region involved the inferior temporal gyrus, the angular gyrus, the precuneus, and the inferior parietal lobule (**Figure 23**). In the interaction region, microglia activation showed a moderate direct correlation with brain hypometabolism, as shown in **Figure 23**.

The interaction region was selected as a seed in the subsequent seed-based interregional correlation analysis, and brain network connectivity was studied in the EOAD and compared with age and sex-matched healthy controls. The brain network identified in the EOAD significantly differed from the brain network in the healthy controls, showing reduced connectivity between the seed region and the frontal cortex (**Figure 24**).

Overall this study, for the first time assessing the relationship between microglia activation and brain glucose metabolism in EOAD patients both at the single subject and at the group level, shows a direct correlation between TSPO overexpression and brain hypometabolism, which has repercussions at the regional and at the long-distance brain network level, altering brain metabolic connectivity.

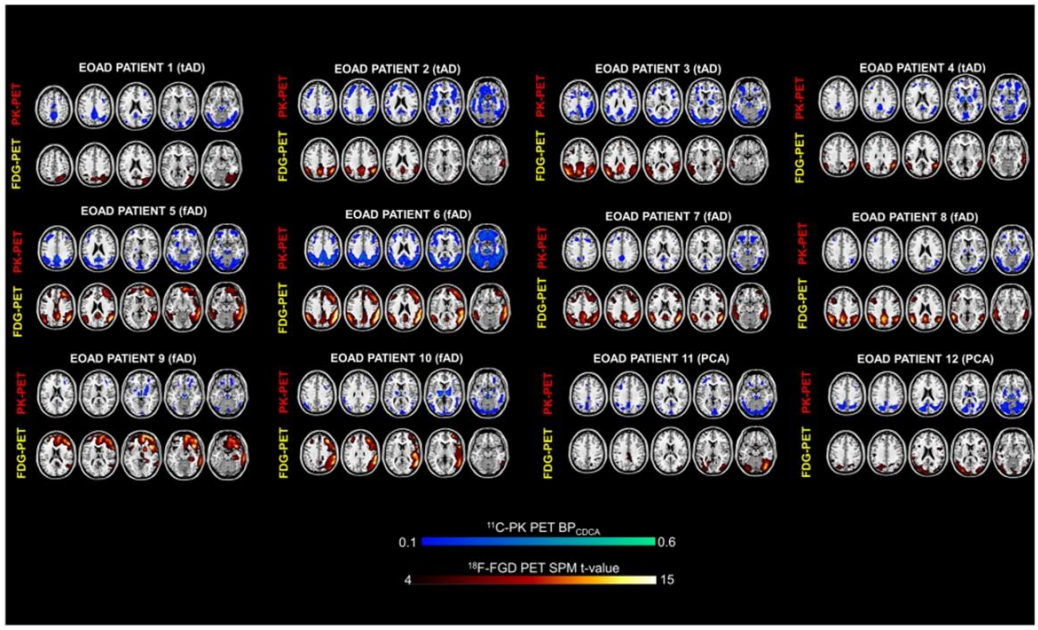


Figure 22. Single-subject maps of [^{18}F]FDG-PET brain hypometabolism and [^{11}C]PK11195-PET TSPO overexpression (source: Tondo *et al.*, 2020b).

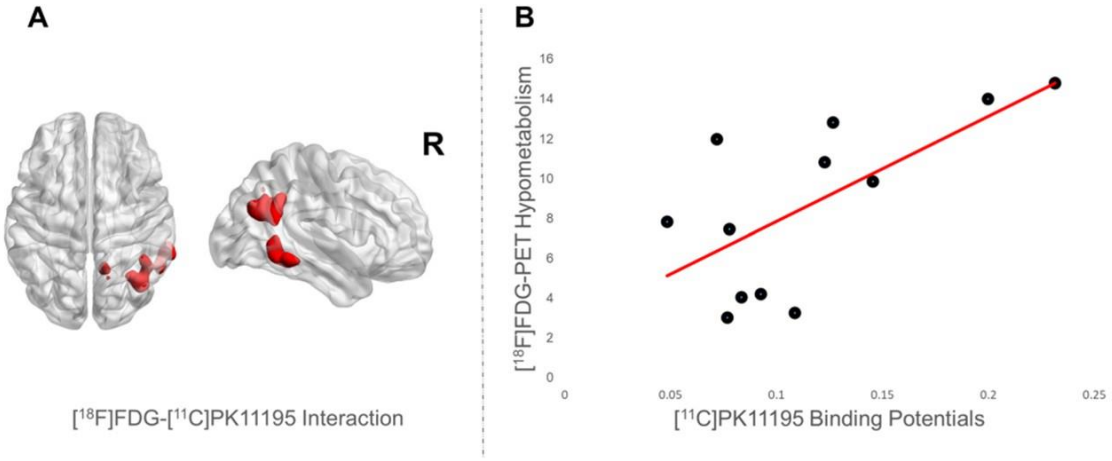


Figure 23. Interaction region between brain hypometabolism and microglia activation, with corresponding direct correlation (source: Tondo *et al.*, 2020b).

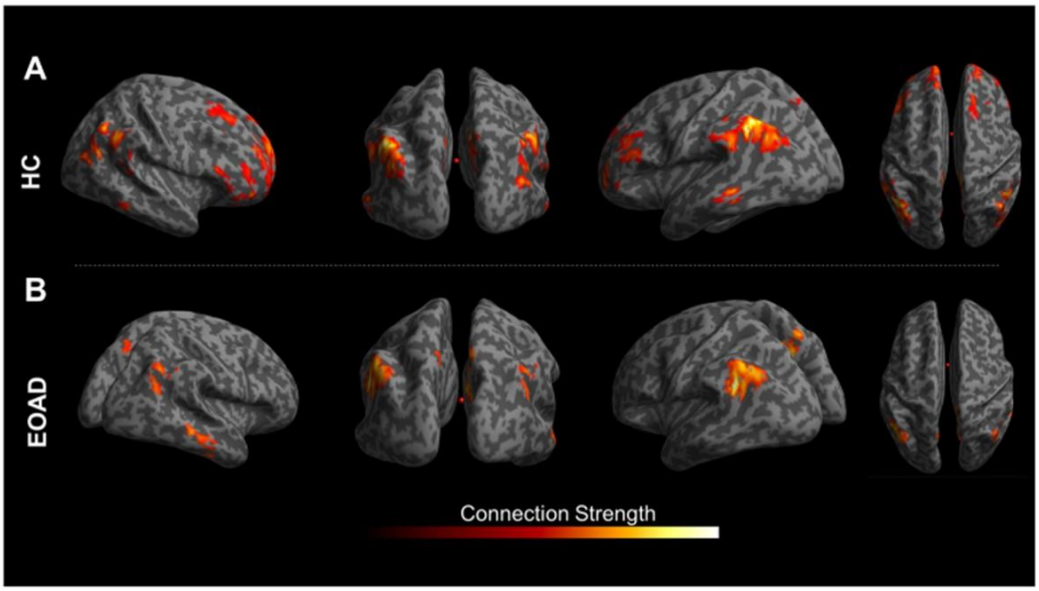


Figure 24. Brain network interregional correlation analysis in the early-onset Alzheimer's disease group, showing network disruption in frontal regions compared with healthy controls connectivity analysis (source: Tondo *et al.*, 2020b).

Study 5. Brain Metabolism and Microglia Activation in Mild Cognitive Impairment: A Combined [^{18}F]FDG and [^{11}C]-(R)-PK11195 PET Study (Tondo et al., J Alzheimers Dis. 2021)

MCI, as an intermediate state between normal aging and dementia, is a heterogeneous condition and includes individuals with different underlying etiologies and variable prognoses (Petersen *et al*, 2001). The amnestic MCI represents the most frequent clinical subtype, and it is often associated with AD pathology, but other less frequent presentations – logopenic, frontal, and visual variants – and other pathological substrates, including FTLD, CBD, cerebrovascular disease, can be possible (Abner *et al*, 2017). MCI represents an intriguing condition to study the effect of neuroinflammatory responses in the neurodegenerative cascade, including individuals whose neurodegenerative changes are still at the earliest phase. The activity of microglia cells is finely modulated at this stage to maintain homeostasis and provide neuroprotection (Comi & Tondo, 2017). Thus, microglia and astrocytic activity in MCI may be enhanced, but both concerning a beneficial and neuroprotective activity or to a deleterious, chronic, and dysregulated response (Liddelow *et al*, 2017).

PET imaging of neuroinflammation can be a precious tool in analyzing neuroinflammatory responses in living individuals in the MCI condition. Previous studies tried to track, *in vivo*, TSPO expression both cross-sectionally and longitudinally in MCI subjects, with some contrasting results. Cagnin and colleagues reported, for the first time in a single MCI individual, TSPO overexpression in temporal regions, even in the absence of clear hypometabolism. Coherently with the absence of signs of neurodegeneration, this subject did not show cognitive deterioration at the follow-up but manifested temporal atrophy corresponding to the region of microglia activation and suggesting an ongoing subclinical process (Cagnin *et al*, 2001). A more recent study investigated microglia activation, brain metabolism, and amyloid deposition in a cohort including AD and PD dementia patients and MCI subjects. The relationship between TSPO expression and brain metabolism appeared inverse in MCI subjects; in addition, microglia activation was associated with amyloid deposition (Fan *et al*, 2015a). Compared with controls, MCI subjects showed an initial peak of microglia activation and subsequent longitudinal reduction, associated with increased amyloid-β deposition, while AD patients showed

increased TSPO expression. These results led to the hypothesis of a double peak of microglia activation along the AD trajectory, with an initial response, possibly protective and aiming to remove senile plaques, in MCI, and a second peak, deleterious, when amyloid-β clearance has failed, in AD (Fan *et al*, 2017). Another study using [^{11}C]PK11195-PET confirmed the initial peak of microglia activation in MCI subjects with a longitudinal increase in amyloid-β deposition and tau tangles formation (Ismail *et al*, 2020). However, a high level of microglia activation can be detected in the presence of high cortical amyloid load but not necessarily correlating with tau burden; this data suggests that neuroinflammation might precede tau tangle deposition (Parbo *et al*, 2018). Currently, no effective treatment is available to stop conversion to dementia. Thus, identifying early biomolecular changes in MCI individuals and clarifying the relationship between different biomarkers may offer a potential therapeutic window to test disease-modifying therapeutic strategies (Cao *et al*, 2018).

The following data have been published in the study "Brain Metabolism and Microglia Activation in Mild Cognitive Impairment: A Combined [^{18}F]FDG and [^{11}C]-(R)-PK11195 PET Study" (Tondo *et al*, 2021). The work evaluated *in vivo* brain metabolism changes and microglia activation in single MCI individuals, using validated single-subject procedures. The MCI sample was investigated by [^{11}C]PK11195-PET as a marker of TSPO overexpression to provide evidence of early microglia activation in neurodegenerative processes, and by [^{18}F]FDG-PET as a marker of neurodegeneration, to provide a single subject classification suggesting the underlying pathology, either AD or other neurodegenerative disorders, and predicting progression with high accuracy (Caminiti *et al*, 2018). We explored the spatial overlap between brain hypometabolism and neuroinflammation. The possible correlation between microglia activation and neuronal dysfunction in the main affected areas was explored following the hypothesis of a detrimental role of microglia activation on neuronal functions, even at the earliest stage of neurodegeneration. Eight subjects fulfilling the Petersen criteria for MCI were enrolled in this study (Petersen, 2004). **Table 7** reports the main demographics and cognitive features of each subject. Single-subject hypometabolism maps were obtained for each participant by testing for relative hypometabolism, on a voxel-by-voxel basis, the subject's scan compared with the large HSR database of N = 112 normal controls (Perani *et al*, 2014a; Della Rosa *et al*, 2014). [^{11}C]PK11195 binding potentials maps were

obtained by subtracting from each participant TSPO-PET scan the non-specific binding extracted from ten healthy controls, used for statistical comparison. A ROIs correlation analysis was used to reveal relationships between [^{18}F]FDG-PET and [^{11}C]PK11195-PET signal. Spatial overlap between brain hypometabolism and microglia activation was estimated. The resulting overlap regions were used to test the strength of the relationship between the two markers, namely to verify whether the strength of the correlation between microglia activation and brain metabolism was linked to the spatial overlap between the two signals. See the "Materials and methods" section for the complete presentation of sample characteristics and neuroimaging analysis.

Subject	Diagnosis and clinical presentation	Sex	Age	MMSE
MCI01	Single-domain, amnestic Mild Cognitive Impairment	M	56	25
MCI02	Multiple-domain, amnestic Mild Cognitive Impairment	M	75	27
MCI03	Multiple-domain, amnestic Mild Cognitive Impairment	M	65	28
MCI04	Multiple-domain, amnestic Mild Cognitive Impairment	M	53	27
MCI05	Single-domain, amnestic Mild Cognitive Impairment	F	73	27
MCI06	Single-domain, amnestic Mild Cognitive Impairment	F	62	29
MCI07	Single-domain, non-amnestic Mild Cognitive Impairment	M	71	27
MCI08	Single-domain, non-amnestic Mild Cognitive Impairment	f	56	27

Table 7. Demographics and clinical features of each Mild Cognitive Impairment subject (adapted from: Tondo et al., 2021).

The single-subject [^{18}F]FDG-PET SPM analysis revealed several hypometabolism patterns, as expected in a MCI sample. Specifically: four subjects showed an AD-like hypometabolism pattern (either typical or atypical AD); one subject had an FTD-like pattern; one subject showed a limbic-predominant (medial-temporal) hypometabolism pattern; one subject had a hypometabolism pattern compatible with CBD; one subject had a negative [^{18}F]FDG-PET scan. [^{11}C]PK11195-PET single-subject analysis showed clusters of increased binding potentials in temporal, parietal, occipital and frontal regions with a strong-to moderate correspondence between hypometabolism topographical distribution and TSPO overexpression in most subjects. **Figure 25** shows the patterns of [^{18}F]FDG-PET brain hypometabolism and [^{11}C]PK11195-PET binding potentials in single individuals. MCI01 is a 56-year-old single-domain amnestic MCI, whose [^{18}F]FDG-PET SPM map shows hypometabolism in temporoparietal cortices, precuneus, and posterior cingulate cortex as in typical AD cases (Perani *et al*, 2016). TSPO expression is higher in the temporoparietal, frontal, occipital, and posterior cingulate

cortex. Temporal and parietal regions show the highest overlap between hypometabolism and TSPO-PET signal. MCI02 is a 75-year-old multidomain amnestic MCI, manifesting impairment in memory and language. [^{18}F]FDG-PET shows hypometabolism in the left superior, middle and inferior temporal gyri and the left inferior and superior parietal lobules, configuring the typical pattern of logopenic variant PPA (Madhavan *et al*, 2013). TSPO overexpression is evident in temporal, parietal, and frontal regions, mainly of the left hemisphere. Temporal and parietal regions show the most considerable overlap between hypometabolism and TSPO-PET signal. MCI03 is a 65-year-old multidomain amnestic MCI with difficulties in memory and visuospatial performances. [^{18}F]FDG-PET underlines hypometabolism in temporoparietal and occipital regions, configuring the typical pattern of PCA (Cerami *et al*, 2015a). TSPO expression is higher, bilaterally, in occipital, temporal, and parietal regions. In addition, occipital, temporal, and parietal regions show the highest overlap between hypometabolism and TSPO overexpression. MCI04 is a 53-year-old multidomain amnestic MCI with memory problems and behavioral disturbances. [^{18}F]FDG-PET SPM map reveals hypometabolism in temporoparietal cortices, precuneus, and posterior cingulate cortex, and in frontal regions, diffusely, as in the frontal AD variant (Woodward *et al*, 2015). TSPO overexpression is limited to frontal, medial-temporal cortices, and subcortical structures. The medial temporal lobe is the region showing the highest overlap between hypometabolism and TSPO expression. MCI05 is a 73-year-old single-domain amnestic MCI. [^{18}F]FDG-PET SPM map shows a focal hypometabolism involving the medial temporal lobe structures, a pattern associated with different underlying etiologies, and overall with long-term clinical stability (Cerami *et al*, 2018). TSPO overexpression is evident in the medial temporal lobe, the lateral temporal cortex, and the orbitofrontal cortex. The medial temporal lobe region shows the most extensive overlap between hypometabolism and TSPO overexpression. MCI06 is a 62-year-old single-domain amnestic MCI with an unremarkable [^{18}F]FDG-PET scan and negligible TSPO expression at the [^{11}C]PK11195-PET. As a consequence, also overlap between hypometabolism and TSPO expression is unremarkable. MCI07 is a 71-year-old single-domain non-amnestic MCI with language impairment associated with behavioral disturbances. [^{18}F]FDG-PET underlines hypometabolism in frontotemporal regions, explicitly involving the perisylvian cortex and the parietal opercula bilaterally, representing a hypometabolism pattern highly

indicative for CBD (Pardini *et al*, 2019). [^{11}C]PK11195-PET shows increased TSPO signal in temporoparietal regions and subcortical structures, including basal ganglia. The most remarkable overlap between hypometabolism and TSPO overexpression is located in the right insula and parietal operculum. MCI08 is a 56-year-old single-domain non-amnestic MCI with behavioral and executive disturbances. [^{18}F]FDG-PET shows hypometabolism in the dorsolateral frontal cortex and the lateral temporal cortex, with an asymmetric distribution involving mainly the right hemisphere, as seen in FTD (Jeong *et al*, 2005). [^{11}C]PK11195-PET shows increased TSPO signal in the frontal, temporal, parietal, occipital cortices, primarily in the right hemisphere. The most significant overlap between hypometabolism and TSPO overexpression is evident in the temporal regions.

By investigating correlation between microglia activation and brain metabolism, a significant inverse correlation was evident between the TSPO and the [^{18}F]FDG-PET signal, suggesting that the clusters of microglia activation were associated with lower metabolic rate. The DICE similarity coefficient used to reveal a topographical overlap between hypometabolic regions and areas of microglia activation confirmed different degrees of overlap in MCI subjects. At the group level, spatial concordance was proportional to the strength of the between signals correlation (**Figure 26**). Overall, the present study shows a close interaction between microglia activation and brain metabolism in MCI individuals with different possible underlying etiologies. Furthermore, these results confirm the presence of an early peak of microglia activation along the dementia continuum, regardless of the cause of neurodegenerative changes.

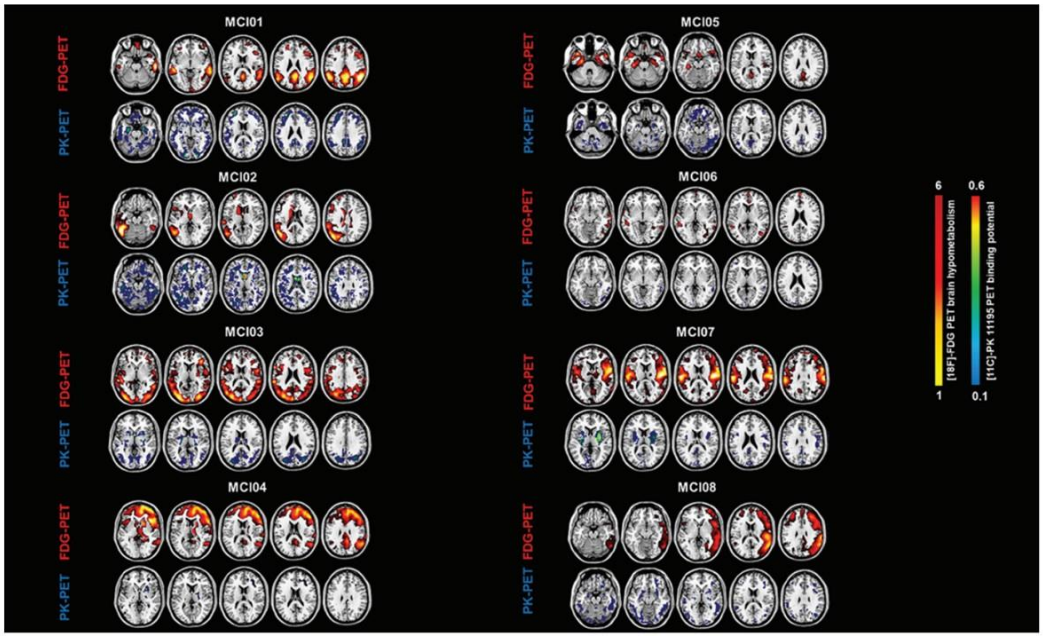


Figure 25. Single-subject patterns of brain hypometabolism and microglia activation (source: Tondo *et al.*, 2021).

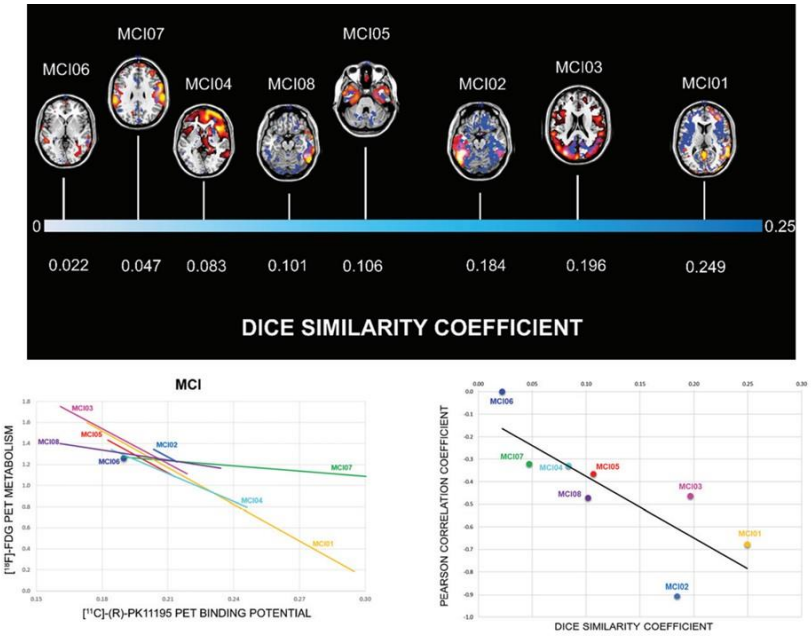


Figure 26. DICE coefficient variability in single Mild Cognitive Impairment subjects with correlation at the single-subject level, based on the strength of overlap between microglia activation and brain hypometabolism (source: Tondo *et al.*, 2021).

DISCUSSION

Dementia is included within the five most burdensome conditions among the elderly worldwide, according to the Global Burden of Disease classification of the World Health Organization (Prince *et al*, 2015). The estimated prevalence of dementia in people over 60 years ranges from 4.7% to 8.7%, and currently, more than 46 million people live with dementia worldwide, and this impressive number is estimated to almost triplicate by 2050 (Prince *et al*, 2015). Age is a major risk factor for dementia, and the increasing life expectancy has been dramatically associated with an increasing prevalence of dementia. Due to its impact, dementia has been defined as a world health priority by the World Health Organization. The need for urgent interventions also arises from the lack of effective disease-modifying therapy.

This dissertation includes studies focusing on identifying and examining reliable diagnostic and prognostic markers in neurodegenerative diseases. The dementia spectrum has been studied in toto, from preclinical to the prodromal and overt dementia phase. Most of the work covers the AD spectrum since AD represents the most frequent etiology of dementia worldwide.

PET imaging techniques represent a unique tool to *in vivo* assess a plethora of biological, pathological, and molecular processes, including synaptic activity, long-distance brain network organization, misfolded protein deposition, and neuroinflammatory responses. The evidence that the pathological deposition of protein aggregates characterizes multiple brain neurodegenerative disorders has led to the concept of proteinopathies, indicating disease spectra sharing the same pathological aggregate (Perani *et al*, 2019b). In the last two decades, the advent of PET tracers to underline cortical amyloid-β and tau pathology has contributed significantly to the *in vivo* diagnosis of AD, intended as a biological entity (Jack Jr *et al*, 2018a). However, the pure biological definition of AD entails some limitations, especially related to the evidence that AD pathology is insufficient to predict inevitable cognitive decline and dementia. (Dubois *et al*, 2021). [^{18}F]FDG-PET, as a marker of synaptic dysfunction, offers the possibility to clarify the spatial and temporal relationship between neurodegeneration and pathological markers of neurodegenerative disease. AD dementia can manifest clinically with the typical (amnestic) and atypical (language, visual, executive) phenotypes. In all

these cases, amyloid-β deposition, the pathological hallmark of AD, is widely distributed across the entire cortex, as *in vivo* confirmed by amyloid-PET, which does not help differentiate among clinical phenotypes (Rabinovici *et al*, 2010). Conversely, intraneuronal tau deposition as visualized by tau-PET is initially confined to brain regions functionally associated with the clinical presentation. When considering [^{18}F]FDG-PET, there is a substantial overlap between brain hypometabolism and tau deposition in typical and atypical AD cases, suggesting that tau deposition is strictly related to regional neuronal and synaptic dysfunction (Dronse *et al*, 2017). Thus, tau-PET can potentially complete [^{18}F]FDG-PET assessment of AD cases providing regional pathophysiological evidence of the etiology of neuronal damage. Nevertheless, the role of amyloid-β pathology in interacting with tau deposition and determining downstream degenerative effects cannot be undervalued. The cortical tau-PET signal measured from AD signature regions is higher with evidence of increased cortical amyloid-β load (Johnson *et al*, 2016). In individuals in the AD continuum, tau-PET can predict cognitive decline better than amyloid-PET positivity if independently analyzed. However, the association of both markers' positivity is a stronger predictor of cognitive impairment, suggesting that neuronal damage is driven by the combined downstream effect of both protein deposits (Aschenbrenner *et al*, 2018). The predictive value of tau-PET is particularly effective in the preclinical dementia phase, overcoming amyloid-PET and brain MRI (Ossenkoppele *et al*, 2021). Nevertheless, the need to associate multiple PET imaging modalities is confirmed in longitudinal studies, showing that amyloid-PET positive MCI and AD may manifest heterogenous tau deposition. On the other hand, a homogeneous decrease in glucose metabolism is observed while disease progress, thus hypometabolism better can track clinical progression (Chiotis *et al*, 2018). A decisive contribution to delineate neurodegenerative processes might be ascribed to PET imaging of neuroinflammation. The co-localization of TSPO overexpression and [^{18}F]FDG-PET decreased signal has been demonstrated in AD (Fan *et al*, 2015a). A correspondence between TSPO overexpression and dysfunctional brain areas responsible for the clinical manifestations has been reported (Kreisl *et al*, 2017). These findings confirm the interplay between microglia activation and neuronal dysfunction. Differently, the relationship between neuroinflammation and amyloid-β and tau deposition still needs further *in vivo* confirmation. Microglia activation has been reported in amyloid-PET positive prodromal

AD without evidence of tau pathology, confirming that microglia activation is an early phenomenon in the neurodegenerative cascade but without conclusively clarifying whether protective or deleterious (Parbo *et al*, 2018). This evidence shows that integrating multiple PET imaging modalities has favored the understanding of neurodegenerative processes. Nonetheless, further studies with larger samples and longitudinal follow-up are vital to clarify the relationship between proteins accumulation, brain metabolism, and neuroinflammation.

The role of [^{18}F]FDG-PET in underlying disease-specific patterns of neurodegenerative diseases already in a preclinical or prodromal stage is of utmost importance, and its value increases if combined with other biomarkers. Study 1 describes preliminary cross-sectional results of the analysis of multiple biomarkers in a large sample of individuals in the MCI condition, aiming to identify the combination of biomarkers that predicts conversion to dementia with the highest accuracy. Single-subject [^{18}F]FDG-PET analysis revealed the presence of multiple sets of subjects grouped by specific brain hypometabolism patterns. AD-like hypometabolism pattern was the most represented. The AD-like group included typical AD cases, showing hypometabolism in the temporoparietal, precuneus, and posterior cingulate cortices and atypical AD variants. These phenotypes are characterized by highly recognizable hypometabolism patterns: a) hypometabolism in the occipital and posterior parietal cortex in the PCA variant; b) asymmetric hypometabolism involving lateral temporoparietal cortices in the logopenic variant PPA; c) temporoparietal and frontal hypometabolism in the frontal AD variant. The link between these specific hypometabolism patterns, the clinical picture, and the corresponding outcome has been consistently reported in the MCI condition (Perani *et al*, 2016; Caminiti *et al*, 2018; Cerami *et al*, 2015c). The combination of [^{18}F]FDG-PET brain metabolism with structural MRI data showed intriguing results. Hippocampal volumetry performed using a validated comparison with a dataset of normal controls (De Francesco *et al*, 2021) showed significant atrophy in the AD-like hypometabolism pattern MCI population in about one-third of cases. The association between AD-like hypometabolism pattern and hippocampal atrophy indicates, in these subjects, a high likelihood of progression to AD dementia. In addition, significant reduction in hippocampal volume is evident with increasing age. These findings are correspondent with previous evidence from the literature. In AD patients has been consistently reported significant

hypometabolism in the temporoparietal regions and the posterior cingulate cortex, associated with decreased hippocampal volume, and the diagnostic accuracy of the two techniques in combination exceeds 90% (Kawachi *et al*, 2006; Dukart *et al*, 2011). The combinate use of these techniques also improves diagnostic performances and prognosis prediction in MCI subjects and allows disease staging (Bauer *et al*, 2018; Benvenutto *et al*, 2018). However, in line with the hypothesis that functional changes (hypometabolism) precede neuronal loss (atrophy) [^{18}F]FDG-PET seems more accurate than MRI in predicting prognosis in MCI subjects (Caminiti *et al*, 2018; Ferrari *et al*, 2019). The employment of quantitative approaches to both modalities may further expand the ability to detect early metabolism and volumetric alterations already in the preclinical dementia AD phase (Gaubert *et al*, 2021; Giannakopoulos *et al*, 2021). Overall, our results underline the importance of integrating [^{18}F]FDG-PET and volumetric MRI data to improve diagnostic and prognostic performances in MCI subjects.

Other interesting findings emerged from the [^{18}F]FDG-PET analysis conducted in study 1. Half of the abnormal scans revealed by the single-subject analysis in the MCI sample were classified as non-AD patterns. Specifically, among non-AD hypometabolism patterns, the medial-temporal hypometabolism pattern (limbic-predominant) was the most frequent, followed by the frontal-like, the DLB, and the MSA patterns. These results confirm that MCI is a heterogeneous condition underlined by several etiologies implying different prognoses and, possibly, different responses to therapy.

The high rate of the limbic-predominant medial temporal hypometabolism pattern causes significant repercussions in the differential diagnosis in the MCI population. Study 2 explicates the features of the MCI subpopulation showing limbic-predominant hypometabolism. These subjects are characterized by amnestic presentation clinically indistinguishable from the typical AD presentation. Conversely to MCI due to AD, limbic-predominant amnestic MCI have a stable clinical profile and do not progress to dementia after long follow-up. The biomarker characterization of the limbic-predominant amnestic MCI group might be misleading, end particularly amyloid-β definition. Our study shows that about half of the limbic-predominant amnestic MCI group present evidence of amyloid-β pathology, but they will not progress to dementia. The large variability in biomarkers alteration expressed in this group suggests the possible presence

of multiple pathological substrates. Neurodegenerative tauopathies, the argyrophilic grain disease, hippocampal sclerosis, PART and LATE may cause the clinical picture in most limbic-predominant amnestic MCI. Especially LATE, may represent the underlying etiology in MCI with suspected non-AD pathology and in subjects with evidence of neurodegenerative changes without concomitant tauopathy (Nelson *et al*, 2019). In addition, LATE and AD neuropathological changes may co-occur, especially in older age. The co-existence of amyloidopathy with other neuropathological substrates hampers the diagnostic workup of the limbic-predominant amnestic MCI, whose amyloid positivity may lead to misclassification and erroneous inclusion in studies enrolling MCI due to AD. In this population, [^{18}F]FDG-PET is the only marker predicting long-term clinical stability. In fact, a limbic-predominant [^{18}F]FDG-PET hypometabolism pattern is associated with 80% possibility of remaining stable after eight years of disease duration compared with the typical AD hypometabolism pattern, which is associated with dementia development in about 90% of cases. Coherently, the accuracy of [^{18}F]FDG-PET in predicting stability or progression in this specific amnestic MCI population reaches 90%. The importance to correctly identify limbic-predominant amnestic MCI derives from the substantial frequency of this pattern, which in study 2 ranges from 16% to 38% depending on the analyzed sample. Previous evidence shows that a high percentage of amnestic MCI remains clinically stable and does not convert to dementia or revert to normal cognition (Roberts *et al*, 2014). The different outcomes suggest that the MCI condition includes different underlying causes (Ganguli *et al*, 2019). We can hypothesize that a significant quote of MCI not progressing to dementia is composed of limbic-predominant amnestic MCI and that [^{18}F]FDG-PET is crucial in screening subjects considered in the prodromal dementia phase.

The heterogeneity of the MCI condition is confirmed in post mortem studies, showing that pure AD pathological changes are responsible for the clinical picture in less than one-quarter of cases. Most MCI cases present mixed AD neuropathological changes, Lewy bodies disease, hippocampal sclerosis, and cerebrovascular pathology (Abner *et al*, 2017). The same heterogeneity has been reported in older adults with memory complaints. Specifically, the neuropathology in individuals with memory complaints includes senile plaques and neurofibrillary tangles, neocortical Lewy bodies, TDP-43 pathology, hippocampal sclerosis, and amyloid angiopathy (Arvanitakis *et al*, 2018). An increasing

number of individuals with SCD search for specialist consultation complaining decline without an objective impairment on formal cognitive testing or neuropsychological performances. SCD are at risk for developing dementia, both AD and non-AD types (Slot *et al*, 2019). However, SCD may be related to various etiologies, including neuropsychiatric disorders, sleep disturbances, drug adverse effects (Balash *et al*, 2013). Consequently, the prognosis of SCD may vary greatly, with some developing dementia, mainly when associated with biomarker evidence of AD pathology, and others remaining stable over a long period (Jessen *et al*, 2020). Due to the poor predictive value, biomarkers investigation is not recommended in cognitively unimpaired individuals in clinical practice, and isolated SCD is considered not specific enough for the AD continuum (Dubois *et al*, 2021). In the research setting, biomarkers investigation in SCD is helping in underlying the pathophysiology of this condition and possibly will provide the possibility to monitor progression and response to therapy. Study 3 analyzes biomarkers of neurodegeneration and pathology and neuropsychological correlates in SCD and pre-MCI, thus in the presence of a subjective decline with respectively absent or subtle cognitive impairment. The [^{18}F]FDG-PET single-subject SPM procedures revealed different hypometabolism patterns, possibly indicating different neurodegenerative etiologies. Specifically, the identified hypometabolism patterns were, by frequency, the frontal-like, the AD-like, the limbic-predominant, the DLB-like. These hypometabolism groups include specific neuropsychological and neuropsychiatric features, such as a higher burden of neuropsychiatric symptoms in the frontal-like group and worse performances in the semantic fluency in the limbic-predominant group, as previously reported (Marra *et al*, 2012). Worthy of note, a high rate of individuals (45%) with SCD and pre-MCI have no neurodegeneration signs, which is a normal [^{18}F]FDG-PET scan. In these individuals, [^{18}F]FDG-PET has a fundamental exclusionary role for actual signs of neurodegeneration. The evidence of normal metabolism is associated with a favorable prognosis and long-term stability (Iaccarino *et al*, 2019). As a confirmation, a recent longitudinal study investigating CSF biomarker positivity in SCD showed a favorable prognosis associated with the absence of biomarkers of pathology and neurodegeneration (Ebenau *et al*, 2020). Therefore, this study confirms that SCD and pre-MCI represent heterogeneous conditions, including neurodegenerative and non-neurodegenerative disorders, and [^{18}F]FDG-PET helps identify the corresponding underlying etiology. In

this case, the comparison with literature data is challenging due to the use of group-analysis approaches in previous works. There are limited [^{18}F]FDG-PET data in SCD and pre-MCI, showing hypometabolism in AD-related regions in SCD compared with controls (Vannini *et al*, 2017; Scheef *et al*, 2012). Hypometabolism in the precuneus has also been correlated with longitudinal decline in memory performance in SCD samples, supporting the possibility to identify very early changes related to the development of dementia. However, group analyses lack specificity and prognostic accuracy, which can be reached with the single-subject approach. This method allowed to identify an AD-like hypometabolism pattern only in 11% of subjects, which is very interesting since SCD and pre-MCI are usually considered preclinical AD stages, preceding the MCI condition (Rabin *et al*, 2017). The reported rate of amyloid positivity in SCD ranges from 12 to 43% (Jansen *et al*, 2015). In our study, an abnormal brain amyloid load was evident in 18% of subjects undergoing amyloid-PET. In about one-third of amyloid-positive cases, there was an AD-like hypometabolism pattern, confirming the neurobiology of AD and the risk to develop AD dementia in a minority of subjects. One-third of subjects showed abnormal amyloid load but a frontal-like hypometabolism pattern, suggesting the possibility of co-pathology, including TDP-43 proteinopathy and cerebral amyloid angiopathy, which can co-exist with AD pathological changes. The last one-third of subjects with abnormal amyloid accumulation showed no brain metabolism abnormalities. These findings feed the debate on the role of amyloid pathology since a considerable percentage of subjects with normal cognition may show brain amyloid deposition, and individuals with abnormal amyloid load and normal [^{18}F]FDG-PET pattern are unlikely to progress to AD dementia, as revealed in a large MCI population (Iaccarino *et al*, 2019). Besides the categorical framework approach, in study 3 a data-driven dimensional approach was applied to capture the presence of neuropsychological and neuropsychiatric dimensions. Different neuropsychological profiles at this stage correlate with neuronal dysfunction in specific brain regions, with crucial differences between SCD and pre-MCI. Three different neuropsychological dimensions, revealed by principal component analysis, correlated with specific metabolic dysfunction: 1) executive/visuomotor, with metabolism in frontal, occipital cortex, and basal ganglia; 2) episodic memory, with metabolism in temporal-parietal regions; 3) visuospatial/constructional, with metabolism in right frontoparietal cortices. These

components are in line with the profile of subtle neuropsychological deficits, involving especially memory and executive functions, recently identified in a large SCD cohort (Wolfsgruber *et al*, 2020). Specifically, the memory profile seems of particular interest. The memory dimension involved subjects whose memory performance correlated with metabolism in the precuneus, cuneus, temporoparietal cortices, cingulate cortex, and the superior and middle frontal regions. These data parallel the distribution of brain hypometabolism in typical AD cases, with additional slight occipital and frontal involvement, confirming that the monitoring of the memory domain is of utmost importance in identifying subjects with possible AD-like risk of progression (Jessen *et al*, 2020). Also, the correlation of neuropsychiatric profiles with brain glucose metabolism showed fascinating correlates. The affective dimensions, including apathy, anxiety, depressive and eating disturbances, were inversely correlated with metabolism in the orbitofrontal cortex, cingulate cortex, and insula. The hyperactive/psychotic dimension, including agitation, irritability, euphoria, aberrant motor behavior, disinhibition, delusions, hallucinations, and night-time sleep disturbances, had a significant inverse correlation with the frontotemporal, insular, and caudate metabolism. These findings are in line with previous reports on subjects with AD dementia. The presence of apathy has been correlated with hypometabolism in the orbitofrontal cortices and anxiety and depression with metabolism in the anterior cingulate and frontal and prefrontal cortices (Ng *et al*, 2019; Ballarini *et al*, 2016). The presence of hypometabolism in the absence of a high amyloid burden at the preclinical stage may suggest that apathy and anhedonia symptoms are signals of neuronal injury driven by other mechanisms, or confer increased vulnerability to AD pathophysiology clinical decline. Given the robust relationship between cerebrovascular disease and affective symptoms and the vulnerability of cognitive control and affective networks to either age-related or vascular changes and FTD (Lee *et al*, 2014), combined etiopathology may contribute to affective neuropsychiatric symptoms in pre-dementia syndromes. Findings of metabolic dysfunctions in hyperactivity/psychotic subsyndromes are mostly limited to subjects in the clinical phase of AD (in particular, delusion, hallucinations, and night-time disturbances are infrequently reported in the early stages). However, sleep disturbances and irritability (which emerges in the dementia prodrome and is associated with similar metabolic changes to agitation) predicted posterior cingulate hypometabolism at two

years (Ng *et al*, 2017). This finding, and the fact that agitation and irritability are associated with metabolic changes reflecting neurodegeneration in regions associated with core AD pathology, suggest that at least the hyperactivity subsyndrome constitutes an early clinical manifestation of AD pathophysiology (Ng *et al*, 2017; Ismail *et al*, 2016). When evaluating SCD and pre-MCI separately, some differences in neuropsychological/neuropsychiatric profiles emerge. The pre-MCI group showed more compromised memory and executive performances, as expected given the inclusion criteria. Accordingly, they showed correlations between memory and executive/visuomotor factors and extended hypometabolism in temporoparietal and frontal regions. On the other hand, SCD subjects drive the correlations between the hyperactive/psychotic factor and widespread metabolism in frontal-temporal regions, suggesting a possible dysfunctional correlate of these neuropsychiatric disturbances (Slavin *et al*, 2010). Notably, considering the whole sample, neuropsychiatric symptoms were highly prevalent, involving 78% of subjects. This aligns with literature data on the preclinical dementia stages, reporting a high prevalence of NPS in SCD populations (Braam *et al*, 2014). The occurrence of neuropsychiatric disturbances may represent a clinical feature per se or a precipitating factor in dementia progression (Ismail *et al*, 2016), pointing out to the importance of a comprehensive clinical, neuropsychological and neurobehavioral, and instrumental approach for the correct identification of different clinical prognostic patterns.

Besides validated markers of neurodegeneration and pathology, the *in vivo* study of neuroinflammation has shed new light on the pathophysiology of aging and neurodegenerative diseases. Currently, the most intriguing hypothesis in neurodegenerative disorders on the role of microglia, the most important immune cells in CNS neuroinflammation, relies on a double response, either protective or deleterious, depending on the disease phase (Hickman *et al*, 2018). The studies included in this dissertation explored the role of neuroinflammation in the overt dementia phase, in prodromal dementia, and both the symptomatic and asymptomatic phase of genetic ALS, aiming to track an ideal trajectory of microglia activation during the development of neurodegenerative changes. All the included studies employed the [^{11}C]PK11195-PET tracer, the most validated radioligands for TSPO, a protein expressed by activated microglia.

Study 4 investigates microglia activation and brain glucose metabolism in EOAD, an AD subtype characterized by frequent atypical onset and fast disease progression. Significant microglia activation is present in widespread cortical areas of AD patients compared with controls. TSPO overexpression has a spatial concordance with brain hypometabolism and primarily in AD signature regions. The direct correlation between TSPO signal and brain hypometabolism in the most affected regions confirms previous evidence that microglia activation directly enhances neurodegenerative changes in brain disorders (Cagnin *et al*, 2001; Edison *et al*, 2008, 2013). The association of microglia activation with brain hypometabolism sustains another hypothesis: TSPO-PET may serve as a specific marker aiding differential diagnosis among AD subtypes, where different patterns of neuroimmune activation may exist according to the clinical phenotype, as shown using a second-generation TSPO tracer (Kreisl *et al*, 2017). Our study shows a fair correspondence between microglia activation and brain metabolism changes, and both signals involve temporoparietal regions in typical AD, posterior regions in PCA, and frontal regions in the frontal AD variant. However, the TSPO signal is rather diffused to several cortical and subcortical regions, sustaining a widespread microglia activation driving neurodegeneration. This aspect is also underlined by the study of the long-distance brain network effect of microglia activation. In our EOAD sample, the network built starting from the microglia/hypometabolism interacting region showed an abnormal organization compared with the healthy controls. Specifically, loss of connectivity between temporoparietal and frontal regions was evident in patients, resembling the functional connectivity alteration in the default mode network recently reported in LOAD, also suggesting a link between connectivity alteration and cognitive decline (Passamonti *et al*, 2019).

The evidence of widespread microglia activation is consistent with the hypothesis that neuroinflammation represents a shared pathological event in neurodegenerative disorders (Ransohoff, 2016). Microglia activation seems to be a common reaction to altered mechanisms regardless of the primary etiology of neurodegeneration. Study 5 aimed to investigate the presence of TSPO overexpression in the MCI condition and the relationship between microglia activation and brain glucose metabolism changes, ideally in a prodromal phase of dementia. The results confirm the interaction between microglia and brain metabolism, which showed topographical overlap and significant inverse

correlations in our MCI sample. The single-subject [^{18}F]FDG-PET analysis confirmed that MCI represents a heterogeneous condition, revealing multiple etiologies including AD, FTD, CBD, and limbic-predominant cases. In the case of identifiable neurodegenerative signs, namely brain hypometabolism, a TSPO overexpression was evident and mirrored metabolism changes, indicating a contribution to neuronal dysfunction. In addition, one MCI subject did not show signs of neurodegeneration. This subject is of particular interest. The MCI subjects with normal [^{18}F]FDG-PET scan also showed negligible microglia activation. An early report presented an MCI subject with loco-regional, temporal microglia activation, and normal brain metabolism. Coherently with the brain metabolism findings, excluding actual neurodegeneration, the MCI subjects did not manifest further cognitive impairment at the follow-up. However, the follow-up MRI showed left temporal atrophy, indicating a possible subclinical process (Cagnin *et al*, 2001). In our case series, the presence of microglia activation is associated with brain hypometabolism, as already reported in MCI due to AD individuals (Fan *et al*, 2015a), while the absence of signs of neurodegeneration also microglia activation is absent. Study 5 shows another interesting aspect of neuroinflammation in neurodegenerative diseases. The spatial overlap between microglia activation and brain glucose hypometabolism was particularly significant in MCI, showing an AD pattern, both typical or atypical. The linear regression analysis showed that the higher the overlap between microglia activation and brain hypometabolism, the stronger was the negative correlation between TSPO overexpression and glucose consumption. As a result, the neuroinflammatory response in MCI due to AD may be more pronounced, as already reported (Parbo *et al*, 2017). Coherently with the double peak hypothesis, microglia can be present as a protective player in the early phase of the degenerative process, such as in MCI. A second, deleterious microglia peak develops due to the failure of protective mechanisms, including protein deposition clearance, synaptic support, and debris phagocytosis (Fan *et al*, 2017). In this second phase of neurodegeneration, microglia cells have lost their protective function. Consequently, therapeutic modulation of neuroinflammatory response should consider the presence of multiple microglia phenotypes (Onuska, 2020). Unfortunately, [^{11}C]PK11195 nor other TSPO PET tracers allow disentangling between different microglia phenotypes. In addition, TSPO-PET and

[^{18}F]FDG-PET detect complementary but different signals, namely deriving from the neuronal and synaptic activity and microglia and astrocytes activity.

Overall, these studies show a widespread microglia activation in the dementia continuum, including the prodromal dementia phase. However, the precise role of neuroinflammation in neurodegeneration is still far from being completely elucidated. In physiology, microglia activity is vital in providing neuronal support, maintaining homeostasis, and favoring the clearance of misfolded protein, including amyloid-β (Comi & Tondo, 2017). In pathology, microglia activation may induce neurodegeneration, fail pathological protein and cellular debris clearance, release pro-inflammatory cytokines, and increase oxidative stress (Heneka *et al*, 2015). The evidence of the correlations between neuroinflammation and hypometabolism and clinical impairment proposes microglia activation as a marker for staging the diseases, monitor disease progression, and prospectively evaluating response to therapy.

Our results imply important therapeutic consideration. The development of therapeutic strategies acting in a timeframe preceding irreversible neurodegeneration is crucial. In *in vitro* and animal models, evidence supports the modulation of neuroinflammatory response as a promising therapeutic target. The *in vivo* modulation of neuroinflammatory responses has been evaluated primarily in AD. The earliest strategy aimed to indiscriminately suppress neuroinflammation in symptomatic AD patients using non-steroidal anti-inflammatory drugs, which showed to be ineffective (Miguel-Álvarez *et al*, 2015). Also, the use of minocycline, an antibiotic with anti-inflammatory properties, failed to preserve cognition in AD patients (Howard *et al*, 2020). These results show that the indiscriminate suppression of neuroinflammation, both the protective and deleterious responses, is not an effective strategy. Multiple compounds are under evaluation, targeting specific molecules or molecular pathways, such as the caspase inhibitors targeting the Nf-κB, anti-TNF, or anti-IL-1 antibodies, which showed promising results in preclinical models (Leng & Edison, 2021). Even more promising are strategies aiming at modulating microglia phenotypes. Several anti-inflammatory cytokines, including IL-2, IL-4, and IL-33, have beneficial effects on AD pathology by modulating microglia activation, but their use *in vivo* is limited, especially by the challenging delivery into the human brain (Alves *et al*, 2017; Fu *et al*, 2016; Zheng *et al*, 2016; Kiyota *et al*, 2010). Several substances may reduce microglia activation, such as the SIRT1 activator

resveratrol, which attenuated the cognitive decline in early AD patients (Moussa *et al*, 2017). In addition, the intervention on lifestyle and risk factors such as obesity, insulin resistance, hypercholesterolemia has been proposed to reduce neuroinflammation in AD (Chen *et al*, 2016a). Dietary balancing, exercise, and vascular risk prevention have shown to be effective in maintaining cognitive function in people at risk for dementia (Ngandu *et al*, 2015). All these hopeful data need further confirmation but encourage research towards a multimodal therapeutic approach, which could overcome criticisms encountered by clinical trials so far.

In June 2021, the Food and Drug Administration approved the utilization of Aducanumab in AD patients, by the Accelerated Approval Pathway, based on the biomarker evidence of a reasonable clinical improvement. Aducanumab is an antibody targeting Amyloid-β aggregates. The efficacy of Aducanumab in slowing AD progression has been evaluated in two phase III clinical trials, the EMERGE and the ENGAGE. Both studies were stopped in 2019 due to the lack of significant clinical benefit. Subsequent analysis showed, in the trial EMERGE, a significant slowing of clinical impairment, evaluated by the CDR, in AD patients treated with Aducanumab compared with patients in placebo. In addition, both trials showed a significant reduction in cerebral amyloidosis in treated patients, as measured by amyloid-PET (Cummings *et al*, 2021a). The Aducanumab approval represented a surprising step forward in the therapeutic approach in AD patients since the previous approval of a drug for treating AD dates back about twenty years (Salloway & Cummings, 2021). However, several controversies emerged before and after the approval. EMERGE results were not replicated in the analysis of the trial ENGAGE. Thus, evidence on the clinical benefit of Aducanumab is based on a single study. The main criticism regards the minimal clinical impairment despite the clear pathological benefit in reducing brain amyloid load. This aspect may be related to several issues. First, Aducanumab and other monoclonal antibodies might reduce senile plaques, but not the more toxic oligomers. Second, reducing brain amyloidosis is not necessarily followed by a cognitive improvement since amyloid-β deposition is not indubitably linked to neurodegeneration, and it can be present also in cognitively unimpaired individuals. The selection of candidates for clinical trials based on the amyloid positivity can hide several limitations, such as the inclusion of patients in an advanced phase of

neurodegeneration, not responding to any therapy, or individuals in the preclinical phase but that will never develop dementia.

The current dissertation discusses all these aspects. The presented studies explored the crucial role of [^{18}F]FDG-PET in underlying disease-specific patterns of neurodegeneration in prodromal and preclinical dementia phases. The integration of multiple imaging modalities, including MRI and amyloid-PET, demonstrated to be vital in characterizing subjects at the initial boundary of the dementia continuum. Presented data are primarily based on cross-sectional analyses, which are essential in defining biomarkers' contribution to the diagnostic process but lack prognostic confirmation. Longitudinal investigation and adequate samples are needed to confirm neuroimaging tools' ability in early diagnosis and disease identification, which are pivotal for the correct design of clinical trials. PET imaging of neuroinflammation adds essential insights into neurodegenerative mechanisms, providing vital information on disease staging and, crucially, offering the possibility to monitor response to therapy. Integrating multimodal diagnostic tools coupled with the multi-target therapeutic approach is a compelling way to tackle neurodegeneration.

MATERIALS AND METHODS

The studies discussed in this dissertation included primarily data analysis of PET scans, namely [^{18}F]FDG-PET, [^{18}F]Florbetaben-PET, and [^{11}C]PK11195-PET. Acquisition procedures, pre-processing methods, and data analysis are shared between studies. Thus, they are fully described at their first appearance, while only study-specific methodological approaches and statistical models are subsequently specified. In addition, other biomarker measures have been employed and are detailly described in each study section.

All PET imaging pre-processing and data analyses have been performed in SPM12 (http://www.fil.ion.ucl.ac.uk/spm/software), implemented in Matlab (MathWorks Inc., Sherborn, MA). All statistical analyses were performed using SPSS version 25.0 (Statistical Package for Social Sciences software, IBM, Armonk, NY, USA).

All studies were approved by the San Raffaele Hospital Ethics Committee and conducted strictly according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants involved in each study.

Study 1. Role of imaging biomarkers in the classification of MCI condition as defined by the NIA-AA criteria. Preliminary data analysis

This study includes data from the Interceptor Project, an Italian multicenter cohort study funded by the Italian Ministry of Health and the Italian Medicines Agency, focusing on the MCI condition. It has enrolled 400 subjects consecutively diagnosed with MCI at 20 Centers for Cognitive Disorders and Dementia distributed on the Italian territory, with the aim to identify the biomarker, or the combination of biomarkers, able to predict with greater accuracy the conversion of MCI to AD dementia after three years of follow-up. A detailed description of the project has been published (Rossini *et al*, 2019).

Participants. The study enrolled 400 MCI subjects fulfilling the MCI criteria as defined by the NIA-AA (Albert *et al*, 2011). MCI subjects with both amnestic and non-amnestic presentations were included. Inclusion criteria were: a) age between 50 and 85 years; b) MMSE corrected score equal or superior to 24/30; c) Clinical Dementia Rating (CDR) global score of 0.5; d) concerns about cognitive changes, expressed as subjective

complaints, or referred by a relative or a clinician; e) defective neuropsychological performance in one cognitive domain (memory, executive function, attention, language, visuospatial function); f) preserved functional autonomy; g) no dementia. Exclusion criteria were: a) history of cerebrovascular disease, alcohol abuse, medical disorders associated with cognitive impairment, including HIV infection; b) neuroimaging evidence of other potential causes of cognitive decline; c) chronic treatment with psychotropic drugs; d) women in reproductive age; e) history of malignancy < 5 years; f) contraindications for MRI, lumbar puncture; g) use of drugs potentially affecting cognitive function.

Included biomarkers. Each biomarker has been evaluated for sensitivity and specificity to predict progression from the MCI condition to dementia. According to the literature data the following biomarkers were selected: MMSE, sensitivity 0.57 and specificity 0.87 (Xu *et al*, 2002); the FCSRT, sensitivity 0.81 and specificity 0.71 (Grande *et al*, 2018); CSF amyloid-β40, amyloid-β42, t-tau, p-tau, and tau/amyloid-β ratios, sensitivity 0.71 and specificity 0.77 (Vos *et al*, 2013); [^{18}F]FDG-PET, sensitivity 0.57 and specificity 0.67 (Herholz *et al*, 2011); Volumetric MRI, sensitivity 0.60 and specificity 0.75 (Frisoni *et al*, 2013); *APOE ε4* genotype, sensitivity 0.52 and specificity 0.66 (Fei & Jianhua, 2013); multiple electroencephalogram for brain connectivity analysis, sensitivity 0.87 and specificity 0.64 (Poil *et al*, 2013).

[^{18}F]FDG-PET image acquisition. According to Standard Operating Procedures, all the [^{18}F]FDG-PET images were acquired on a 3D PET scanner in the recruiting centers, allowing to perform a reliable SPM analysis (Presotto *et al*, 2018a). The subject is asked to attend the PET Unit before the scan for preliminary evaluation and blood glucose dosage, whose upper limit is considered 140 mg/dL to guarantee correct scan execution. Then, the subject receives the intravenous injection of the tracer, a precalculated [^{18}F]FDG dose rate of 3.7 MBq/kg (with a minimum of 185 MBq), while resting and lying supine in a quiet room, free from auditory and visual stimuli. Emission image is acquired in 3D mode 45 min after intravenous tracer injection. Scan duration is 15 minutes, and the acquisition is performed in a dynamic mode by three consecutive five-minutes extended frames. This procedure allows for correcting any microscopic head movements or eventually to discard macroscopic movements. Image reconstruction follows an ordered subset expectation maximization (OSEM) algorithm. The

simultaneous acquisition of a computed tomography scan is used for co-registration and attenuation correction. Scatter correction is applied with software integrated into each scanner. Images and row data are uploaded on a dedicated database platform to be further analyzed at the Nuclear Medicine Unit of the San Raffaele Hospital, Milan, Italy.

[^{18}F]FDG-PET image pre-processing. All analyses are performed in SPM12, implemented in Matlab (MathWorks Inc., Sherborn, MA). The images are placed in the standard Montreal Neurological Institute (MNI) reference space using a Dementia-Specific [^{18}F]FDG-PET template for SPM software (Della Rosa *et al*, 2014). This template has been shown to provide a higher degree of accuracy for spatial normalization of FDG-PET scans and higher statistical sensitivity at the single-subject level. The optimized single-subject method is based on a highly robust statistical comparison of the single-subject scan with a large number of healthy controls (N = 112) (Perani *et al*, 2014a). The normalized images are smoothed (Full-Width at Half Maximum: 8mm) and enters a statistical comparison using a two-sample t-test with the dataset of scans from normal controls (that underwent the same pre-processing), with the statistical threshold set at p = 0.05, family-wise error-corrected, considering significant clusters including more than 100 voxels. The analysis includes age as a covariate, considering that aging is associated with brain glucose metabolism changes. The SPM method allows the identification of single-subject disease-specific brain hypometabolism patterns, with crucial diagnostic ability and prognostic accuracy (Perani *et al*, 2014a, 2016).

MRI acquisition and processing. Brain MRI was acquired in the recruiting centers on a 1.5 or 3 Tesla scan. During the acquisition, the subject must be awake with eyes closed. The acquisition protocol includes conventional sequences and T1-weighted volumetric imaging. MRI images are uploaded to the online database portal and processed by the expert MRI center at the Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy. Volumetric measures are obtained applying the FreeSurfer version 6.0 (http://surfer.nmr.mgh.harvard.edu/) algorithm pipelines for automatic hippocampal segmentations, voxel-based morphometry, gray matter density, and cortical thickness, according to validate procedures (Frisoni *et al*, 2015). Freesurfer quantifies hippocampal volumetry (in mm^3), and results from the volumetric analysis are compared with those obtained from a sample of cognitively normal people from the ADNI database.

Imaging analysis. A total of 369 [^{18}F]FDG-PET has been analyzed. The single-subject method resulted in a subject-specific map of brain hypometabolism, visually rated by four experts in neuroimaging, blind to each other and to clinical information. The expert raters showed a high agreement (Cohen's k: 0.89) and classified subjects into different patterns: a) normal brain metabolism, no signs of neurodegeneration; b) AD-like pattern, hypometabolism in temporoparietal regions, posterior cingulate cortex, and precuneus (Cerami *et al*, 2015c; Perani *et al*, 2016); c) frontal-like pattern, variable hypometabolism involving the frontal lobes (Cerami *et al*, 2015c); d) DLB-like pattern, temporoparietal and occipital hypometabolism (Caminiti *et al*, 2017); e) MSA-like pattern, hypometabolism in basal ganglia or cerebellum (Caminiti *et al*, 2017); f) limbic-predominant or medial-temporal pattern (Cerami *et al*, 2018).

Hippocampal volumetry. MCI subjects showing the AD-like pattern were further analyzed employing the MRI hippocampal volumetry protocol. A total of 120 MRI images were available. Left and right hippocampal volumetry has been analyzed and compared with the ADNI dataset of normal controls to detect subjects below the fifth percentile, individuals at high risk for developing cognitive decline (De Francesco *et al*, 2021). In addition, MRI data of the 120 MCI has been compared with a dataset of 40 individuals with SCD showing normal neuropsychological performances (data acquired from a different study, see description in Study 3), to underline differences in hippocampal volumetry.

Study 2. Biomarker-based stability in limbic-predominant amnestic mild cognitive impairment (Tondo et al., Eur J Neurol. 2020a)

Participants. Subjects were retrospectively included from two datasets: a) San Raffaele Hospital, Milan, Italy (screened subjects N = 280, included amnestic MCI N = 60); b) ADNI dataset (adni.loni.usc.edu), screening the ADNI1, ADNI-GO, and ADNI-2 phases (screened subjects N = 818, included amnestic MCI N = 62). Inclusion criteria were: a) amnestic MCI diagnosis according to Petersen criteria (Petersen 2004); b) disease duration ≥ four years, to guarantee an appropriate timeframe to detect progression from the amnestic MCI condition to dementia; c) available baseline CSF analysis; d) available baseline [^{18}F]FDG-PET scan. The results of the optimized SPM single-subject

procedure were used to screen the sample further. Only MCI subjects showing one of the two specific hypometabolism patterns were included in this work: a) medial-temporal hypometabolism (limbic-predominant pattern); b) temporoparietal, posterior cingulate, and precuneus hypometabolism (AD-like pattern). The sample selection strategy, led to the inclusion of 80 subjects with amnestic MCI and limbic-predominant hypometabolism pattern, and 62 amnestic MCI with AD-like hypometabolism pattern.

Clinical, cognitive, and functional evaluations. The assessment included the MMSE and the CDR for evaluating the global cognitive status, Instrumental Activities of Daily Living (IADL), and Functional Assessment Questionnaire (FAQ). All tests were performed at the baseline and follow-up evaluation. The Index of progression was calculated to evaluate cognitive decline between baseline and follow-up, using the formula: MMSE score at follow-up – MMSE score at baseline/years of follow-up. The score indicates the number of MMSE points lost per year.

[^{18}F]FDG-PET imaging acquisition, pre-processing, and analysis. Image acquisition was performed at the Nuclear Medicine Unit of the San Raffaele Hospital, Milan, Italy, following the European Association of Nuclear Medicine guidelines (Varrone *et al*, 2009), and in the recruiting centers for the ADNI cohort, according to the validated acquisition procedure described in the "ADNI PET technical procedures manual, version 9.5"(http://adni.loni.usc.edu/wpcontent/uploads/2010/09/PET-Tech_Procedures_Manual_v9.5.pdf). Images pre-processing was conducted as described above and the optimized single-subject SPM procedure was employed to obtain single-subject specific hypometabolism maps (see the section "Materials and methods" Study 1, for a complete description). ADNI imaged were downloaded and pre-processed to obtain a single NIFTI file containing the last 15 min of PET acquisition, then merged to obtain a single file that entered the SPM single-subject procedure.

CSF analysis. CSF levels of amyloid-β42, t-tau, p-tau were evaluated singularly and in combination as amyloid-β/tau ratios and dichotomously classified (normal/abnormal) according to the manufacturer's protocol and literature recommendations (Sjogren *et al*, 2001; Shaw *et al*, 2009).

ATN classification. The available biomarkers were used to classify the amnestic MCI into subjects with AD-profile (A+T-N+ and A+T+N+) and subjects with non-AD-profile (A-T-N+ and A-T+N+). CSF amyloid-β defined "A" positivity; CSF p-tau defined "T"

positivity; [^{18}F]FDG-PET was considered a marker of neuronal injury, thus was present, as an inclusion criterion, in all amnestic MCI subjects.

Statistical analysis. Differences in clinical, cognitive, ATN classification in the amnestic MCI cohorts were examined by the one-way analysis of variance (ANOVA) and the Kruskal–Wallis test, with the statistical threshold set at $p < 0.05$. The predictive value of [^{18}F]FDG-PET SPM hypometabolism patterns for conversion or stability in the amnestic MCI cohort and hazard ratios were estimated by a Cox proportional model (threshold set at $p < 0.05$), and the prognostic performance of the single-subject SPM maps was evaluated by using measures of sensitivity, specificity, and accuracy. Receiver-operating characteristic curve analysis was employed to determine the optimal cutoff discriminating stable amnestic MCI and converters. The predictive value of other variables (CSF measures, ATN classification) was tested in multiple logistic regression models, with diagnosis at follow-up as the dependent variable. In addition, the confounding effect of age, sex, education, and MMSE adjusted score at baseline were evaluated.

Data presented in this study have been published in the work "Biomarker-based stability in limbic-predominant amnestic mild cognitive impairment" (Tondo *et al*, 2020a)

Study 3. Brain metabolism and amyloid load in individuals with subjective cognitive decline or pre-Mild Cognitive Impairment

This study included individuals enrolled in the prospective multi-center Network-AD project (AD-NET-02346784). The project involved N = 459 subjects in the dementia continuum, from the SCD stage to the overt dementia phase. The project aimed to develop and validate operational research diagnostic criteria, integrating clinical, neuropsychological, CSF, and imaging measures, for early diagnosis of AD in Memory Clinics.

Participants. In the current study, individuals in the preclinical dementia phase were included. Clinical and neuropsychological assessments performed at baseline discriminated between SCD and pre-MCI. SCD had self-reported cognitive complaints assessed by specialist clinicians through interviews. SCD subjects (N = 49) had a self-

experienced persistent decline in cognitive capacity but normal age/education adjusted performances on standardized cognitive tests, and a Clinical Dementia Rating (CDR) score = 0. Pre-MCI (N = 56) presented CDR scores = 0.5, and no impairment in objective neuropsychological tests, or a CDR score = 0, but very mild impairment (within 1.5 standard deviation of normal scores) in neuropsychological tests. All included subjects had baseline neuropsychological and neuropsychiatric evaluation and baseline [^{18}F]FDG-PET. A subgroup (N = 60) also had baseline amyloid-PET.

Clinical, cognitive, neuropsychological, and neuropsychiatric assessment. The MMSE evaluated global cognitive status. The neuropsychological assessment included tests for evaluating memory (FCSRT immediate and delayed free recall, Rey-Osterrieth figure recall), language (fluencies), visuospatial and constructive abilities (Rey-Osterrieth figure copy and cube-copying drawing), attention and executive functions (Trail Making Tets). The neuropsychiatric evaluation included the Neuropsychiatric Inventory and the Starkstein Apathy Scale. For subsequent analysis, Neuropsychiatric Inventory scores were clustered into four independent dimensions as follows: (1) affective (anxiety and depression); (2) apathetic (apathy, eating and appetite changes); (3) hyperactivity (agitation/aggression, irritability, euphoria/elation, aberrant motor behavior, and disinhibition); (4) psychotic (delusions, hallucinations, and night-time sleep disturbances) (Aalten *et al*, 2007). The score for each neuropsychiatric dimension was defined as the sum score of the included NPI symptoms (Ballarini *et al*, 2016).

[^{18}F]FDG-PET imaging acquisition, preprocessing, and analysis. All subjects underwent [^{18}F]FDG-PET scans acquired in the participating centers. The [^{18}F]FDG-PET acquisition procedures were conformed to the European Association of Nuclear Medicine guidelines (Varrone *et al*, 2009). The subsequent analyses were all performed at the Nuclear Medicine Unit of the San Raffaele Hospital using validated procedures, which guarantee high reproducibility of results, also using images from different scans (Presotto *et al*, 2018a). The complete procedure is described in Study 1.

[^{18}F]Florbetaben PET image acquisition, preprocessing, and analysis. Amyloid-PET scan was performed in N = 60 subjects, using [^{18}F]Florbetaben (Neuraceq, Piramal). Data analysis was conducted at the Nuclear Medicine of the San Raffaele Hospital. A validated preprocessing pipeline allowed to extract global cortical [^{18}F]Florbetaben Standard Uptake Value ratio (SUVr) using normalization data based on the low-dose computed

tomography (CT) scan contextually acquired with the PET-scan (Presotto *et al*, 2018b). To calculate cortical amyloid burden, six ROIs were defined using the AAL atlas 20 through the Wake Forest University PickAtlas toolbox for SPM12. These regions included the dorsolateral and medial frontal cortex, cingulum, precuneus, inferior and superior parietal lobules, lateral occipital cortex, lateral temporal cortex. In addition, images were scaled to the activity of the cerebellar grey matter, used as the reference region (Barthel *et al*, 2015; Catafau *et al*, 2016). Finally, the global cortical amyloid load was calculated as the average computed from the six ROIs. A cutoff of 1.45 was considered for classifying subjects in amyloid-positive (Aβ+), with global cortical amyloid load higher than the chosen cutoff, or amyloid negative (Aβ-), global cortical SUVr equal or below the selected cutoff (Ong *et al*, 2015).

Principal Component Analyses. Two principal component analyses were applied in the whole sample using orthogonal varimax rotation to explore the underlying dimensions of variation in the patients' neuropsychological and neuropsychiatric measures. All the scores were converted to z-scores based on the results of the whole cohort. The neuropsychological and neuropsychiatric measures entered two different principal component analyses to collapse the data into composite principal component analyses scores. The correlation matrix was used for the extraction of components. The adequacy of the sample size of both PCAs was determined utilizing the Kaiser-Meyer-Olkin measure of sampling adequacy and Bartlett's test of sphericity. Component loadings > 0.5 were considered meaningful, and component scores were computed using the regression method. To investigate whether these factors were differentially expressed by SCD and pre-MCI groups, the regression scores of the PCA factors were compared using one-way ANOVA. To identify the correlations between brain metabolism and the PCA factors, the z-scores of the tests grouped in the PCA factors were used as independent variables in voxel-wise multiple regression analyses together with global mean scaled metabolic rate as dependent variables (both in the whole subjects' group and separately in the SCD and the pre-MCI). Age was entered as a nuisance variable. For each PCA, a multivariate correlation matrix was computed using SPM12, running in MATLAB. The statistical threshold was set at p-value <0.05, with Kep≥100 voxels. The neuropsychological variables entered a PCA to collapse the test z-scores into composite PCA factors. Sample size was acceptable (KMO= 0.561), and correlations were

sufficiently large (Bartlett's test = 553.115, p<0.000). According to the PCA, three components captured 62.69% of the variance, and they were interpreted as the best dimensional representation of the full dataset. The neuropsychiatric variables entered a PCA to collapse the test z-scores into composite PCA factors. Sample size was acceptable (KMO= 0.622), and correlations were sufficiently large (Bartlett's test = 27.39, p<0.002). Two components captured 54.65% of the variance.

Statistical analysis. The ANOVA test was used for normally distributed variables, and the Mann–Whitney U test for non-parametric variables was used to test differences in neuropsychological and neuropsychiatric features between SCD and pre-MCI subgroups.

Study 4. The combined effects of microglia activation and brain glucose hypometabolism in early-onset Alzheimer's disease (Tondo et al., Alzheimers Res Ther. 2020b)

The following data have been published in the study "The combined effects of microglia activation and brain glucose hypometabolism in early-onset Alzheimer's disease" (Tondo *et al*, 2020b)

Participants. The study involved twelve patients with EOAD whose symptoms onset occurred before 65 years. Data acquisition was performed at the IRRCS San Raffaele Hospital, Milan, Italy. All participants underwent cognitive and neuropsychological evaluations, [^{18}F]FDG-PET, [^{11}C]PK11195-PET, CSF analysis for confirming AD pathology. Nine healthy controls underwent [^{11}C]PK11195-PET, following the same procedure of EOAD patients, and were used for statistical comparison in TSPO-PET analysis.

Cognitive and Neuropsychological evaluations. Clinical, cognitive and neuropsychological assessments were carried out at the Departments of Neurology and the Department of Rehabilitation and Functional Recovery, San Raffaele Hospital, Milan, Italy. The cognitive and neuropsychological assessment included the MMSE and tests for evaluating memory, language, attention, executive and visuospatial abilities, specifically: the Token Test; semantic and phonemic verbal fluencies; the backward and forward span; the Rey Auditory Verbal Learning Test; the Rey-Osterrieth figure (copy and recall); the

attentive matrices; the Stroop test; the Raven progressive matrices test; the clock drawing test.

[^{18}F]FDG-PET acquisition, pre-processing and data analysis. [^{18}F]FDG-PET scans were acquired at the Nuclear Medicine Unit, San Raffaele Hospital, Milan, Italy. The acquisition protocol followed standard procedures (Varrone *et al*, 2009). Pre-processing and data analysis employed the validated and standardized pipeline of the single-subject SPM method, allowing to obtain single-subject SPM hypometabolism maps (Perani *et al*, 2014a) (see Study 1). In addition, we conducted a group-level analysis to compare the twelve EOAD [^{18}F]FDG-PET with the same large database of N = 112 controls used for the single-subject analysis, after standard pre-processing including co-registration, normalization to the MNI space, and subsequent smoothing. Statistical threshold was set at $p < 0.001$ uncorrected, considering significant clusters containing more than 100 voxels.

[^{11}C]PK11195-PET acquisition. [^{11}C]PK11195-PET was performed at the Nuclear Medicine Unit, San Raffaele Hospital, Milan, Italy. [^{11}C]PK11195 synthesis was performed in the Cyclotron Unit of the Nuclear Medicine of the San Raffaele Hospital, obtaining a radiochemical purity > 95% (Matarrese *et al*, 2001). The [^{11}C]PK11195 injected dose was 380 ± 37 MBq. The acquisition protocol required a dynamic PET scan lasting 58 minutes and including 15 frames (6× 30 s/2× 1 min/1× 3 min/3× 5 min/2× 10 min/1× 15 min). Trans axial images were reconstructed using a Shepp–Logan filter (cutoff 5 mm) in the trans axial plane and a Shepp–Logan filter (cutoff 8.5 mm) in the axial direction. Corrections for attenuation artifacts, radioactive decay, scatter, and movement was applied. Images analysis was performed using the SPM12 software.

[^{11}C]PK11195 pre-processing and data analysis. A receptor parametric mapping procedure was used to extract [^{11}C]PK11195 binding potentials (Gunn *et al*, 1997). The heterogeneous distribution of the [^{11}C]PK11195 across the whole brain hampers the definition of a reference region. For this reason, clustering method have been developed for [^{11}C]PK11195-PET analysis. In this study, images were analyzed using the curve distance clustering algorithm, an adaptation of the validated SuperVised Clustering algorithm (Turkheimer *et al*, 2007; Presotto *et al*, 2015). The curve distance clustering algorithm estimates the similarity of the time-activity curve of each voxel within four predefined curves (i.e., tracer delivery in blood, white matter, gray matter with non-

specific binding, gray matter with high specific binding). This method allows the selection of a cluster of voxels devoid of specific uptake, which can be used as a pseudo-reference region for the subsequent parametric analysis (Presotto *et al*, 2015). The obtained maps were normalized to the standard MNI space using a specific PET template, after masking to remove extracranial components (Iaccarino *et al*, 2018b), then smoothed (8 mm FWHM). Voxel-wise statistical differences between the EOAD and the healthy controls groups were analyzed at the single-subject and group-levels. Analyses were run with SPM12, using age as nuisance covariate, with the statistical threshold set at $p < 0.01$ (uncorrected for multiple comparisons), considering significant clusters containing more than 100 voxels.

Interaction analysis. The [^{18}F]FDG and [^{11}C]PK11195-PET voxel-wise group analysis resulted in two maps expressing hypometabolism and TSPO overexpression, respectively. Both maps were binarized and superimposed in an interaction analysis performed with IMcalc in the SPM software applying the following formula: Interaction mask = [^{18}F]FDG hypometabolism map × [^{11}C]PK11195 TSPO map.

Correlation analysis. We tested correlation between cerebral glucose metabolic rate uptake and [^{11}C]PK11195 binding potentials extracting values from the interaction mask for each EOAD patient (Spearman's rho correlation, $p < 0.05$).

Brain network analysis. Brain metabolic connectivity was explored by the seed-based interregional correlation analysis, selecting as a seed the interaction region, namely the region showing the highest anatomical and functional overlap between microglia activation and brain hypometabolism. Metabolic connectivity analysis was also performed in a group of 20 age- and sex-matched healthy controls for comparison, using the same seed.

Study 5. Brain Metabolism and Microglia Activation in Mild Cognitive Impairment: A Combined [^{18}F]FDG and [^{11}C]-(R)-PK11195 PET Study (Tondo et al., J Alzheimers Dis. 2021)

Participants. In the current study, eight subjects fulfilling the Petersen criteria for MCI (Petersen, 2004) were enrolled. All participants underwent neurological, cognitive (MMSE), and complete neuropsychological evaluation, [^{18}F]FDG-PET and

[^{11}C]PK11195-PET. In addition, ten healthy controls were available from an in-house [^{11}C]PK11195-PET database, and were used for statistical comparison in TSPO analysis.

[^{18}F]FDG-PET acquisition, pre-processing and data analysis. All [^{18}F]FDG-PET scans were performed at the Nuclear Medicine Unit, San Raffaele Hospital, Milan, Italy, and at the Nuclear Medicine, Spedali Civili Brescia, Brescia, Italy, using identical acquisition and reconstruction protocols (see Study 1). [^{18}F]FDG-PET data analysis was performed using the SPM12 software, following the optimized and validated single-subject procedure (see Study 1).

[^{11}C]PK11195-PET acquisition, pre-processing and data analysis. All [^{11}C]PK11195-PET scans were performed at the Nuclear Medicine Unit, San Raffaele Hospital, Milan, Italy, within six months from the execution of the [^{18}F]FDG-PET. The acquisition protocol has been described in Study 4. Mean [^{11}C]PK11195 injected dose was 339 ± 46 MBq for MCI and 375 ± 64 MBq for controls. [^{11}C]PK11195 binding potentials were extracted implementing the previously described receptor parametric mapping procedure (see Study 4). A non-specific [^{11}C]PK11195 binding potential map was obtained by group analysis in the healthy control group. The non-specific TSPO map was subtracted by each [^{11}C]PK11195 MCI map. Images analysis was performed using the SPM12 software.

Correlation analyses. The WFU PickAtlas toolbox (Maldjian *et al*, 2003) implemented in Matlab, using the automated anatomical labeling atlas (Tzourio-Mazoyer *et al*, 2002), was used to define the following regions of interest: frontal lobe, occipital lobe, parietal lobe, temporal lobe, insula, hippocampus, parahippocampal region, amygdala, caudate, putamen, pallidum, and thalamus. Correlation analyses were performed between [^{11}C]PK11195 binding potentials and cerebral glucose metabolic rate uptake, extracted from each ROI, using the toolkit REX, implemented in MATLAB (Pearson's correlation, $p < 0.05$). To provide a measure of the spatial overlap between microglia activation and brain glucose hypometabolism, we calculated the Dice similarity coefficient between the single-subject binarized [^{18}F]FDG-PET maps and the [^{11}C]PK11195 maps. The dice coefficient measures the overlap between two binarized maps, using the formula $DICE = 2 * (A \cap B)\ A + B$. $A \cap B$ indicated the number of elements common to the two masks. A + B indicated the sum of all elements present both in A and B. Dice is equal to 1 if A and B show the highest concordance, assuming the

same logical value in every voxel. Dice is equal to 0 if A and B show the lowest (null) concordance. Dice was calculated for the whole brain and each ROI.

Linear regression analysis. A linear regression analysis, considering Dice similarity coefficients as independent variables and the between-signals Pearson's correlation coefficients as the dependent variable, was calculated. This statistical design demonstrated whether the correlation between microglia activation and brain metabolism was due to the spatial overlap between the two signals.

Data presented in this study have been published in the work "Brain Metabolism and Microglia Activation in Mild Cognitive Impairment: A Combined [^{18}F]FDG and [^{11}C]-(R)-PK11195 PET Study" (Tondo *et al*, 2021).

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