Early and differential diagnosis of Alzheimer's disease

By

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In loving memory of my mother

Table or Content

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Summary

This thesis focuses on early and differential diagnosis of Alzheimer's disease (AD) using multimodality imaging, cognitive measures and artificial intelligence methods of classifications. The findings suggest that measures of semantic free recall and immediate learning are better at detecting preclinical and prodromal cases of Alzheimer's disease (AD). Similarly, neuronal loss in the ventral tegmental area (VTA) appears a very early occurrence in AD, influences hippocampal volume loss and memory performance and can predict future cognitive decline in ageing. Overall the VTA appears to be a better proxy of disease than amyloidosis, since this latter is not associated with clinical symptoms but it is strongly associated with age. In addition, measures of region to region functional connectivity in temporal cortex were found to be better at distinguishing healthy cases from cases of prodromal AD than measures of impaired activity in the posterior cingulate cortex. In fact, variance in activity in this region appeared to be associated largely with age rather than explained by disease severity as expressed by hippocampal volume.

Articles included in this thesis

1. De Marco M, Beltrachini L, Biancardi A, Frangi AF, Venneri A. Machine-learning Support to Individual Diagnosis of Mild Cognitive Impairment Using Multimodal MRI and Cognitive Assessments. Alzheimer Dis Assoc Disord. 2017 Oct-Dec;31:278-286. doi: 10.1097/WAD.0000000000000208.
2. De Marco M, Venneri A. Volume and connectivity of the Ventral Tegmental Area are linked to neurocognitive signatures of Alzheimer's disease in humans. Journal of Alzheimer Disease, 2018 doi: 10.3233/JAD-171018
3. Venneri A, De Marco M. Reduced monoaminergic nuclei MRI signal detectable in pre-symptomatic older adults with future memory decline. Scientific Reports 2020 10 (1), 1-11
4. Venneri A, Mitolo M, Beltrachini L, Varma S, Della Pieta' C, Jahn-Carta C, Frangi AF, De Marco M. Beyond episodic memory: Semantic processing as independent predictor of hippocampal/perirhinal volume in aging and Mild Cognitive Impairment due to Alzheimer's Disease. Neuropsychology, 2019 Feb 18. doi: 10.1037/neu0000534.
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Candidate's contribution to the articles included in this thesis

1. The candidate's contribution to this article is as follow: obtained funding, study conception, study design, clinical and imaging data collection, planning of data analyses and supervision of data analyses, drafting and finalising of manuscript.
2. 3. 4. 5. The candidate's contribution to these articles is as follow: obtained funding, studies conception, studies design, clinical and imaging data collection (except for article 3, Venneri and De Marco 2020, where data were sourced from the Alzheimer's Disease Neuroimaging Initiative freely available database), planning of data analyses and supervision of data analyses, drafting and finalising of manuscripts.

Introduction

1. Alzheimer's Disease

Alzheimer's disease (AD) is the commonnest cause of dementia and accounts for up to 70% of all dementia cases. Dementia is classed as a major neurological disorder by the Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V). This disorder has a considerable impact on society and a major economic impact on social and health care systems. It affects primarily people who are in the sixth, seventh and eighth decade of life and its prevalence doubles every five years after the age of 65 (Fiest et al., 2016). It is estimated that there are about 40 million people living with dementia worldwide, mostly amongst the over 60 years of age. It is also forecasted that these figures would double every 20 years up to 2050 (Ferri et al., 2005). These projections are strongly linked with the fact that there is a progressive increase in the geriatric population since with advanced medical care people are living longer and more people are now reaching the at-risk age range. Indeed according to the Centre for Disease Control, the number of over 65 people will increase from 420 millions in 2000 to 1 billion by 2030 (Centers for Disease & Prevention, 2003). Dementia prevalence is very low in the under 50 year old range, less than 1 case in 4000, and about 30% of these cases are attributable to AD (Lambert et al., 2014).

Contrary to common thinking, research has highlighted that prevalence and incidence of dementia have decreased in the last two decades (Matthews et al., 2013), owing primarily to the beneficial effects of increased years of education and better control of risk factors and cardiovascular pathologies (Langa et al., 2017;

Schrijvers et al., 2012; Wu et al., 2016). Again because more people are living well into the at-risk age, the number of AD cases is, however, increasing and it is estimated that each year there are between 5 to 7 millions new cases of AD worldwide.

AD is a pathological process that is strongly age related. Its incidence increases exponentially with age, but plateaus at around the age of 85 (M. Robinson, Lee, & Hane, 2017). In such an epidemiological context, it is quite clear that AD represents a crucial burden for health systems across the globe, and it will be even more of a burden in the developing world where numbers are said to raise even more steeply than in the developed world. It is, therefore, not surprising that AD remains the centre of attention for scientific research in the international scientific community.

1.1 Aetiopathogenesis

AD is a neurodegenerative condition, the aetiopathogenesis of which acknowledges several mechanisms and pathological hypotheses. Since 1906-1907, when the disease was first observed and described by Alois Alzheimer (Alzheimer, 1907), up to now several pathological processes have been put forward, the real impact of which on the disease, at least for some, has been questioned lately.

Historically, the main pathological pillars that have been acknowledged as major players in the genesis of AD are the accumulation of β-amyloid plaques in the brain extracellular environment and of neurofibrillary tangles of hyperphosphorylated tau protein in the intracellular environment. In more recent years, however, new theories and hypotheses have been put forward that have allowed research to have a better, yet still incomplete, picture of this disease.

1.1.1 The amyloid cascade hypothesis

The Amyloid β protein (Aβ) was isolated and sequenced for the first time in 1984 (Glenner & Wong, 1984). However, since AD acquired an independed nosologic status, Aβ plaques have been credited with a key role in the aetiopathogenesis of this disease. The amyloid cascade hypothesis was first formulated in the early 90s (J. Hardy & Allsop, 1991; J. A. Hardy & Higgins, 1992). However, this hypothesis provides a good disease model that explains and clarifies the molecular bases of the autosomic dominant genetic forms of AD (*early-onset* AD), but not of the sporadic form of this disease (*late-onset*), for which, despite extensive research, the mechanism that leads to abnormal accumulation of Aβ remains elusive and unclear. Some tentative hypotheses have been put forward suggesting that there might be an abnormal production of Aβ and/or an alteration of mechanisms that are deputed to its clearance, most likely determined by an association of specific genetic polymorphisms and the process of ageing (Mawuenyega et al., 2010) (Tanzi & Bertram, 2005) (Selkoe & Hardy, 2016). More specifically, the soluble Aβ42 isoform has been found to be more likely to result in oligomers and fibril formation than the more abundantly produced Aβ40 isoform (Jan, Gokce, Luthi-Carter, & Lashuel, 2008). There also is evidence suggesting that there is impaired drainage along para-arterial pathways with ageing that can be linked to cerebral amyloid angiopathy and to parenchymal Aβ deposition (Diem et al., 2016; Weller, Preston, Subash, & Carare, 2009).

Less than 10% of AD cases are genetically trasmitted via an autosomic dominant route, with complete penetrance. The most frequently observed is a mutation

involving the gene coding the amyloid precursor protein (APP) on chromosome 21. The APP is a transmembrane protein, the cleavege of which by γ-secretase leads to the production of extracellular Aβ (Kang et al., 1987). Numerous mutations of APP have been identified and these all lead to alterations of APP cleavage and consequent increase of the Aβ42 isoform in comparison to other Aβ isoforms (Scheuner et al., 1996).

Another gene that is susceptible to mutations causing a genetically determined form of the disease is that coding for preseniline 1 (*PSEN1*). This gene is located on chromosome 14 and codes for a polytropic mebrane, a component of the γ-secretase complex responsabile for Aβ cleavage. Mutations of the *PSEN1* gene lead to alterations of the way in which γ-secretase cuts Aβ, leading to an altered Aβ40/Aβ42 ratio in the extracellular environment (De Strooper et al., 1998).

Preseniline 2 (*PSEN2*) has a role that is analogous to preseniline 1. The gene coding for it is located on chromosome 1. As for the similarity of function, mutations of the *PSEN2* gene lead to similar aberrations as those that result from mutations of the *PSEN1* gene and also lead to similar outcome (Citron et al., 1997).

Aβ may have both protective and toxic functions, depending on the types of isoform. Its Aβ40 isoform is protective against excessive amyloid deposition, and therapeutic strategies targeting this isoform can even worsen the pathological outlook of AD (J. Kim et al., 2007). As for the neurotoxic properties of Aβ, its harmful action on neurons can be exerted in many different ways and on different targets. It has been suggested that Aβ in its oligomeric forms might exert its toxic effect on cellular membranes by acting on channels and stability of the phospholipid bilayer, on synapses by altering their morphology and function, and on

mitochondria by causing substantial increase in production of oxigen free radicals (OFR) (Reiss, Arain, Stecker, Siegart, & Kasselman, 2018). Furthermore, Aβ appears to have a role in fostering tau protein hyperphosphorylation and its subsequent deposition into neurofibrillary tangles, at the intracellular level (Selkoe & Hardy, 2016).

Although the amyloid cascade has been a cornerstone of AD pathogenesis for numerous years (see **Figure 1** for a graphical illustration of its suggested pathways in genetic and sporadic AD), with a large body of studies providing supporting evidence, in more recent years doubts have been raised by scientists on the effective role of Aβ in the aetiopathogenesis of sporadic AD. Doubts have been mainly reinforced by the outcome of a large number of failed clinical trials of products that targeted amyloid β deposits in the brain (Mehta, Jackson, Paul, Shi, & Sabbagh, 2017), and these doubts remain despite recent controversial approval by the USA Food and Drug Administration of one of these products, despite the weak and controversial results of the relevant clinical trials (Walsh, Merrick, Milne, & Brayne, 2021). These failures have propelled Aβ to the centre of a heated scientific debate, in which the dogmatic view of the amyloid cascade as the core aetiopathological mechanism of AD is rejected or questioned, advocating that research should also focus on alternative or parallel disease models in an attempt to discover novel prospective therapeutic targets (Herrup, 2015).

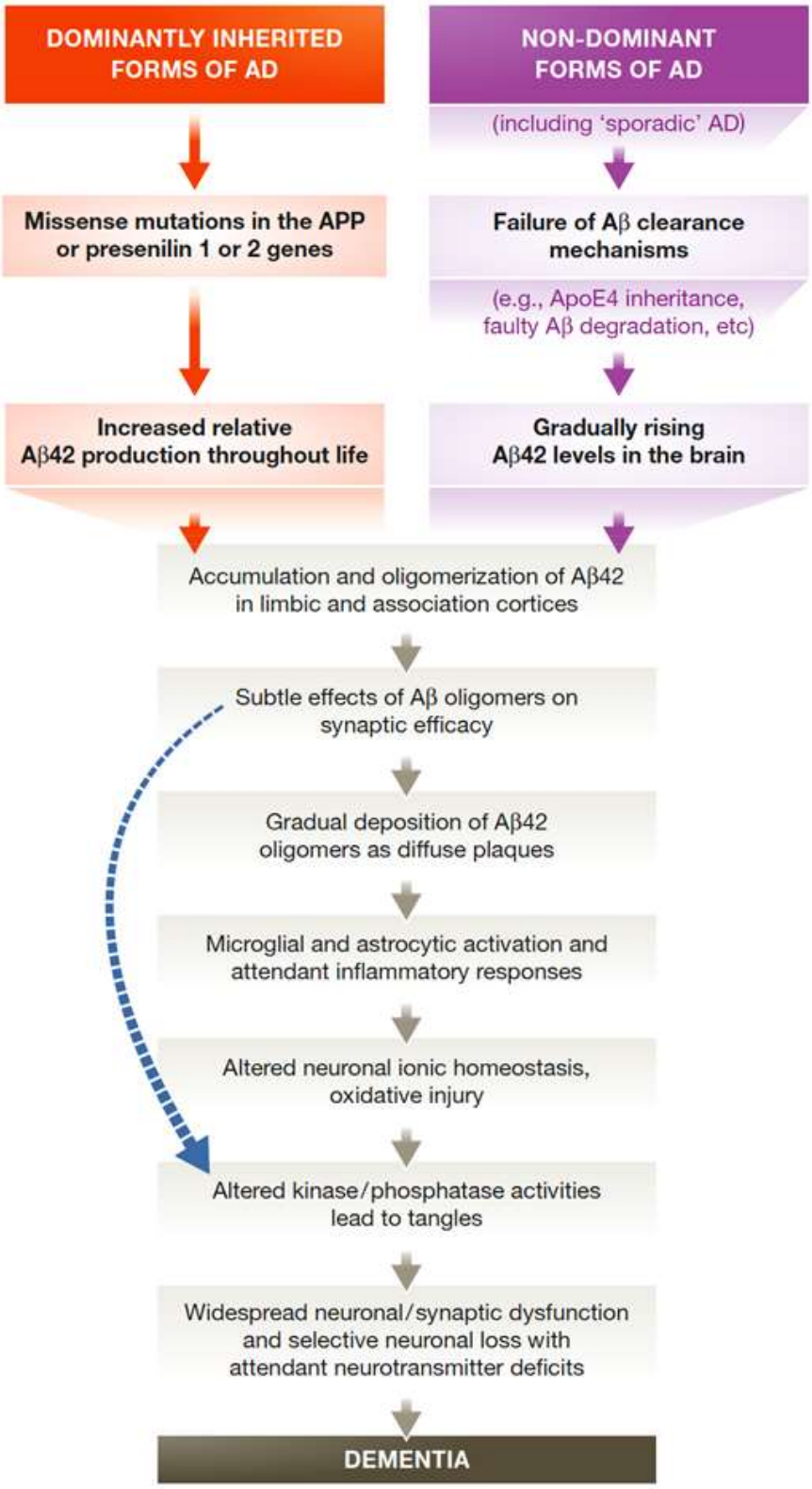

Figure 1. Role of the amyloid cascade in the aetiopathogenesis of Alzheimer's disease. (Selkoe & Hardy, 2016) (*reproduced under the Creative Commons licence*)

1.1.2 The role of the tau protein

From a physiological point of view, the tau protein, encoded by the *MAPT* gene on chromosome 17, has a crucial role in intracellular trafficking and in maintaining the cytoskeletal structure of neurones. However, when this protein undergoes hyperphosphorylation, its link with microtubuli fails, increasing the amount of free intracellular tau. This latter form of the protein aggregates forming neurofibrillary tangles (NFT) and tau aggregates in neuritic processes around amyloid plaques that form neuritic plaques that, together with Aβ plaques are considered the typical hallmarks of AD. Studies have suggested that there might be a causal link between tau malfunction, dysfunction of its kinase and phosphatase in the context of AD neurodegeneration. This hypothesis has emerged from evidence showing that treatments targeting the above mentioned enzymes are also able to decrease levels of hyperphosphorylated tau and NFT deposition (Ballatore, Lee, & Trojanowski, 2007).

Mounting evidence has also progressively emerged indicating that Aβ might also foster tau protein phosphorylation by acting on different pathways such as inflammation, kinases and phosphatases deregulation, axonal transport and tau degradation (Blurton-Jones & Laferla, 2006). A causal link between Aβ and tau is supported by logical foundations grounded in the observation that in the autosomal dominant forms of AD that are linked to mutations affecting Aβ equilibrium, Aβ alterations are followed by hyperphosphorilated tau depositions; in contrast, alterations due to tau mutations are not followed by Aβ accumulations (Selkoe & Hardy, 2016).

There is, however, evidence in support of the independence of these two pathological pathways. Studies that have analysed the timing of deposition of NFT and of Aβ plaques have shown that tau protein deposits are found in the entorhinal and perirhinal cortex, and some have even suggested that the earliest site of tau aggregation is the brainstem, while the initial Aβ deposits are found at first in the neocortex (Braak & Braak, 1991; Thal, Rub, Orantes, & Braak, 2002). Furthermore, NTFs' load, contrary to amyloid plaques' load, correlates with the severity of cognitive decline. This observation has been taken as confirmatory evidence that the tau protein pathway has a direct effect on cognition and fosters neurodegeneration (Bejanin et al., 2017). There is no similar evidence in support of a direct link between Aβ deposit accumulations and severity of cognitive decline or neurodegeneration (Jack, Wiste, Knopman, et al., 2014; Jack, Wiste, Weigand, et al., 2014).

NFTs contributes to neuronal death via malfunction of microtubuli. Such a deficit determines a halting of axonal transport and traffic, and in turn compromises the equilibrium and cytoscheletric stability of neurons, leading to neuronal death (Ballatore et al., 2007). This, however, is only one of the potential routes to neuronal loss, a late occurrance in the pathological cascade, as other mechanisms have been postulated, including synaptic dysfunction and loss that play an important role, but the actual mechanism leading to neuronal death remains still unclear.

1.1.3 The cholinergic hypothesis

The link between dysfuntion of cholinergic pathways [and acetylcholine (ACh)] and AD became acknowledged by researchers in the field when Whitehouse

provided evidence of significant loss of magnocellular neurons in the nucleus basalis of Meynert (NbM) in patients with AD (Whitehouse, Price, Clark, Coyle, & DeLong, 1981). The evidence from these earlier studies provided one of the first and most developed therapeutic targets for AD. Findings showed that increasing ACh half-life at synaptic level led to improvements in cognitive performance. Earlier evidence led to the development of acetylcholinesterase inhibitors (AChEI) and clinical trials showed significant improvements resulting from this therapeutic approach (J. Birks, Grimley Evans, Iakovidou, & Tsolaki, 2000; J. S. Birks, Melzer, & Beppu, 2000; Blesa et al., 2003).

In clinical practice, AchEI treatments are reported to have only modest efficacy and are considered mostly symptomatic (J. L. Cummings, 2003; Hampel et al., 2017). There are, however, several studies advocating that the effects of some of these treatments go beyond symptomatic improvements and argue that they might play a key role in interfering with disease progression at least in part, leading to a delay/slowing down of disease progression in AD (Venneri & Lane, 2009; Venneri, McGeown, & Shanks, 2009; Venneri et al., 2002).

1.1.4 The vascular hypothesis

Considerable evidence has accumulated that points at a role of cerebrovascular dysfunction and vascular comorbidity in the pathogenesis of late onset sporadic AD (Di Marco et al., 2015). There is evidence that age-related and vascular comorbidity-related chronic hypoperfusion leads to alteration of the brain hemodynamic homeostasis resulting in impaired signalling among neurons and astrocytes and even damage to the blood brain barrier (de la Torre, 2012; Di

Marco et al., 2015). This evidence has led to the two-hit hypothesis of AD, according to which its 'first hit' (i.e. the consequences of chronic cerebral hypoperfusion) can then initiate a pathological cascade that induces deposition of AD pathology via a non amyloidogenic route. This latter process acts as a 'second hit' fostering accumulation of Aβ peptides due to increased production and diminished clearance of Aβ that together increase neurotoxicity and accelerate neurodegeneration (Sweeney, Sagare, & Zlokovic, 2018; Zlokovic, 2011).

1.1.5 The mitochondrial/bioenergetic hypothesis

One additional pathophysiological pathway that has been proposed to explain the aetiopathogenesis of sporadic late onset AD is the mitochondrial cascade hypothesis (Swerdlow & Khan, 2004). This hypothesis maintains that mitochondrial dysfunction related to ageing triggers processes that foster amyloid pathology by affecting the expression of APP, its processing and production of Aβ. Supporters of this hypothesis suggest, therefore, that typical biomarkers of AD seem to detect ageing related processes, with the implication that treatment to clear amyloid from the brain would have little if any effect on treating the disease (Swerdlow, Burns, & Khan, 2014). Additional support for mitochondrial dysfunction as a potential pathway involved in the genesis of sporadic AD comes from recent studies that have highlighted how energetic and respiratory mitochondrial deficits are detected in early AD and correlate with cognitive dysfunction (Bell et al., 2021; Bell et al., 2020)

1.2 Risk factors

1.2.1 Genetic risk factors

Many genes have been implicated in sporadic AD. Of these the most compelling evidence has been provided by studies of the apolipoprotein E (*APOE*) gene on chromosome 19. There are several allelic forms of this gene. The standard and most frequent form is the *APOE* ε3 allelic variant. It is widely recognised that the *APOE* ε4 allelic variant represents the most significant genetic risk factor for sporadic late onset AD. The presence of this allele increases the risk of developing the disease of 2-3 times in those individuals carrying a single copy of the allelic variant and of 12-14 times for those homozygous for the allele (Farrer et al., 1997). In contrast the ε2 allelic variant of the *APOE* gene appears to be protective. Furthermore, the ε4 allele is also said to be linked to an earlier age of onset of AD (Lambert et al., 2014; Sando et al., 2008; Saunders et al., 1993). The ε4 allele exerts a negative impact on the integrity of the ageing nervous system, fostering neurotoxic mechanisms (Mahley, Weisgraber, & Huang, 2006). Several models have been proposed to explain how the *APOE* ε4 allele fosters the onset of AD. These include alterations of intracellular trafficking, inhibition of neuritic growth, and even facilitation of tau protein phosphorylation and increase of amyloid plaque deposition (Mahley et al., 2006). Imaging studies have highlighted how the presence of the ε4 allele determines alterations in functional connectivity in the *Default Mode Network* (DMN) of the brain, both in healthy individuals and in the early stage of AD (Filippini et al., 2009). A number of studies has investigated the

effect of the *APOE* polymorphism on the structural and functional neural architecture of healthy adults (Filippini et al., 2009; Heise et al., 2014; Lu et al., 2017; Machulda et al., 2011; Nao et al., 2017; Sheline et al., 2010; Song et al., 2015; Westlye, Lundervold, Rootwelt, Lundervold, & Westlye, 2011; Yang et al., 2014). The neuroimaging ε4-related phenotype emerging is that of a neural system with reduced computational resources. Similar studies in patients with a clinical diagnosis of AD confirm this pattern. The volume and functional connectivity of the hippocampus are significantly reduced in patients who carry the ε4 isoform compared with non-carriers of comparable clinical profiles (De Marco et al., 2017; J. Wang et al., 2015), and so is functional connectivity within the DMN (K. Wang et al., 2007).

APOE genetic status, however, is only one of the possible risk factors and more recent advancements in the molecular characterisation of individuals with sporadic AD have identified about 20 additional genes that contribute to a polygenic risk score estimated to cover about 90% of the genetic risk in AD (Escott-Price, Myers, Huentelman, & Hardy, 2017; Escott-Price, Shoai, Pither, Williams, & Hardy, 2017), of which, however, the *APOE* ε4 allelic variant explains the greatest proportion of risk and the greatest proportion of regional neurodegeneration as expressed by measures of atrophy. Indeed recent evidence from voxel wise analysis of the neural signature of a polygenic hazard score in patients with mild cognitive impairment defined clinically or in people who had biomarker evidence of amyloid pathology showed that, after stratification for *APOE* status, the poligenic hazard score was predictive of volume loss only in non carriers of the *APOE* ε4 allelic variant (De Marco et al., 2020). A more recent review lists over 30 genes that have

been found to be associated either with an increased risk or protection against AD (Kamboh, 2018). These additional genes, identified via genome wide association studies, are involved in pathogenic pathways such as inflammation, cholesterol, etc. One particular polymorphism, the *TREM2* polymorphism has been specifically linked to microglia activation and has been found to play a crucial pathogenic role (Guerreiro & Hardy, 2013; R. Guerreiro et al., 2013; R. J. Guerreiro et al., 2013; Qin et al., 2021).

1.2.2 Demographic risk factors

Age is amongst the most consistently reported demographic risk factors for AD and also the one with the largest influence. Indeed, most cases of AD occur after the age of 60, with prevalence raising with increasing age but levelling off after the age of 85 (Ritchie & Kildea, 1995). This makes AD an age-related disease (Ritchie & Kildea, 1995). The prevalence of AD dementia is estimated at about 1% at age around 60 years, but raises up to 25% at age 85. Earlier studies that have looked at dementia without differentiating by aetiology have given catastrophic predictions about rates of prevalence in the oldest range of the population (figures range from 62% to 77% at around 95 years, suggesting an exponential increase in prevalence from age 60 (Jorm, Korten, & Henderson, 1987). These studies and others are strongly flawed, however, as they included only a limited number of people in the oldest age ranges. If adequate adjustments are made to take into account the small numbers of people included in the oldest age range, what is observed is a different scenario. Rather than observing an exponential increase, the prevalence rates of dementia look like a flattened S curve that indicates that in the very old, prevalence

rates begin to fall and by the age of 95, prevalence rates are estimated to be around 40% (Ritchie & Kildea, 1995). Additional research also found a flattening of prevalence rates in the oldest age range, demonstrating that for every five-year increase, the incidence of Alzheimer's disease triples before age 64, doubles before age 75 but then increases in the range of 1.5 times are seen by age 85 (Gao, Hendrie, Hall, & Hui, 1998). Given this evidence, a general consensus has formed that AD should be conceived as 'age-related' - referring to a specific age range, rather than 'ageing-related' - an inevitable consequence of ageing.

In addition to age, gender differences have also been reported. Research indicates that women have an increased risk of AD with an odds-risk ratio of 1.56 (95% CI: 1.16-2.10) relative to men (Gao et al., 1998). A possible explanation for this type of finding might be related to the effects of hormonal changes in women as they get older. This hypothesis is supported by evidence that hormone replacement therapy appeared to lead to a 54% reduction in risk of women in a 16 year longitudinal study (Kawas et al., 1997). Other authors have also suggested that gender differences may be due to males having an increased risk of vascular impairment and, therefore, being less likely to develop a pure form of AD (McCullagh, Craig, McIllroy, & Passmore, 2001).

In addition to the factors reviewed above, low educational and occupational attainment have also been found to contribute to increasing the risk of developing AD (Ott et al., 1995; Ott et al., 1999). A study of elderly residents in rural Italy found that having fewer than three years of education was associated with an odds ratio of 1.8 (95% CI 1.0 – 3.4) of developing AD (Katzman, 1993). The main explanation is related to the role that education and high professional attainment

have on building a cognitive reserve that would allow for more long lasting compensation and mitigation of the effects on cognition due to neuropathological changes (McCullagh et al., 2001). There are several studies indicating that active reserve such that conferred by education delayes the onset of symptoms (Staff, Murray, Deary, & Whalley, 2004). Education can also foster better brain perfusion resulting in higher levels of regional cerebral blood flow in certain regions of the brain (Chiu, Lee, Hsiao, & Pai, 2004). There also is published evidence pointing to the contrary, with studies linking education and schooling to a faster rate of cognitive decline in patients with AD. For example, Roselli et al. (2009) observed that patients with more than 8 years of schooling had a more rapid decline in MMSE scores than patients who had fewer than 8 years of education. However, if taken as a whole these studies indicate that, although higher education delays the clinical manifestation of the disease, once brain damage has reached a certain threshold, decline manifests with a steeper rate, indicating that cognitive reserve allows individuals to delay onset of symptoms, but eventually accumulation of pathology overcomes the initial protective effect. The apparent advantage, therefore, most likely confers a delay of clinical manifestations, as people with higher educational attainament normally have higher intellectual skills and are probably more able to use compensatory strategies.

More recently studies have been carried out investigating the role of diabetes mellitus as a risk factor for AD. There is evidence indicating that diabetes contributes to increasing the risk of AD significantly, indicating that both high levels of glucose as well as abnormal insulin concentration play a crucial role (Hersi et al., 2017; Profenno, Porsteinsson, & Faraone, 2010). There are studies, however,

that suggest that the presence of diabetes, although a risk factor for AD dementia and vascular dementia, is not linked to higher levels of AD neuropathology (Alafuzoff, Aho, Helisalmi, Mannermaa, & Soininen, 2009). There is accumulating evidence that obesity is also a risk factor for AD and some studies suggest that a high Body Mass Index (BMI) represents a risk factor for the onset of AD (Lee et al., 2010), while other studies highlight that significant variations in BMI, indipendently of direction of change, do also contribute to increaseing the risk of AD (Ravona-Springer, Schnaider-Beeri, & Goldbourt, 2013). There is additional evidence that a BMI lower than normal is also associated with an increased risk of AD (Chen et al., 2010).

Despite evidence that numerous factors have an impact on the risk of developing AD (Hersi et al., 2017), the presence of the *APOE* ε4 allele is still the strongest predictor of future onset of AD.

2. Diagnosis

2.1 Clinical symptoms of AD

There is wide consensous that the pathogenic mechanisms of AD preceed its clinical symptoms and manifestation by several decades (Jack, Wiste, Weigand, et al., 2014). This time interval represents the extensive preclinical stage of AD. Research efforts have, therefore, intensified to discover clinical features that might be indicative of potential AD pathology before individuals are aware or manifest clinically relevant symptoms (Sperling et al., 2011).

The earliest clinically detectable symptoms manifest in a prodromal phase of AD that is normally referred to as mild cognitive impairment (MCI). People who experience MCI due to AD have a significantly higher risk of developing dementia. MCI is characterised by a mild cognitive decline that is, however, more severe than that expected for age based on an individual's educational attainment, but not sufficiently severe as to affect everyday activities of daily living or social skills (Petersen & Morris, 2005; Petersen et al., 1999). Cognitive decline experienced by people with MCI can affect a single cognitive domain or multiple cognitive domains. Most people with MCI who progress to AD dementia experience episodic memory decline as the earliest symptom and if this is the only affected domain, it is referred to as amnestic MCI (a-MCI). When cognitive decline involves additional domains such as language, executive functions, attention, visuospatial abilities, etc., it is referred to as amnestic multiple domain MCI (amnestic md-MCI). If cognitive decline is experienced in one cognitive domain other than memory, it is referred to as non-amnestic MCI (na-MCI). Finally if cognitive decline is experienced in multiple cognitive domains but with sparing of episodic memory, it is referred to as non amnestic multiple domain MCI (non amnestic md-MCI) (Petersen, 2000a, 2000b, 2000c; Petersen, 2004; Petersen et al., 2004).

Typically, the initial clinical manifestation of AD neurodegeneration is a decline in anterograde episodic memory, with individuals struggling to recall recent events, and to learn and retain new pieces of information. After an initial prodromal phase with mainly amnestic difficulties, additional decline is observed in other cognitive domains such as attention, executive functions, praxis, visuospatial skills and language (McKhann et al., 2011). There might also be cases in whom AD presents

in an atypical way, with decline observed in cognitive domains other than memory at first (Murray et al., 2011). These variants have been found to have language symptoms at onset (logopenic variant) (Bonner, Ash, & Grossman, 2010; Henry & Gorno-Tempini, 2010), visuospatial deficits (posterior cortical atrophy) (Feher, Mahurin, Inbody, & Pirozzolo, 1989), or executive and behavioural deficits (frontal variant) (Johnson, Head, Kim, Starr, & Cotman, 1999). Whether these variant presentations or atypical forms of AD are true instances of the disease or clinical manifestations of alternative forms of neurodegenerations or mixed forms sharing some traits with AD has been questioned by some experts. It is now widely acknowledged, however, that these atypical forms are detectable, and their specific aetiology thought to be broadly aligned with AD pathology (Ala, Frey, & Clark, 1996; Johnson et al., 1999; Mesulam et al., 2008; Rabinovici et al., 2008)

In addition to cognitive symptoms, neuropsychiatric or behavioural symptoms can also be observed as the initial symptoms heralding the onset of the disease. These symptoms can also be present at the prodromal MCI phase (Sheikh et al., 2018). The most common behavioural or psychiatric manifestation are apathy, depression, psychosis and agitation. Their prevalence is all but negligible and greatly impacts patients' and their caregivers' quality of life, in addition to impacting patients' prognostic outlook (Zhao et al., 2016). There is some additional more recent evidence that AD can manifest clinically in a prodromal stage with non cognitive symptoms, with a mild behavioural syndrome as its earliest manifestation (Ismail et al., 2016). Finally, some evidence has also emerged that AD can manifest clinically with impairment in awareness of its earliest signs (Cacciamani et al., 2017).

2.2 The neuropsychological profile of AD

Neuropsychological assessment still plays an essential and irreplaceable role in the clinical diagnosis of AD. AD diagnosis is still based on clinical criteria (McKhann et al., 2011). Although in research contexts there has been a tendency to favour a biological diagnosis of AD following published guidelines (Jack et al., 2018), more recent guidelines suggest that a biological diagnosis is insufficient and should be accompanied by clinical evidence of symptoms (Dubois et al., 2021).

The approach to neuropsychological assessment for detecting cognitive decline in AD is that of using extensive test batteries including validated tests to assess various aspects of cognition. Ideally, these batteries should include tests that have normative data valid for the relevant population, that have been stardardised and for which norms stratified for age and education are available (Lezak, Howieson, Bigler, & Tranel, 2012). This ideal scenario is only available in limited specialised centres. The great majority of people do not have access to extensive testing and only brief screening scales are used. These screening scales lack the necessary sensitivity to detect the earliest subtle manifestations of the disease.

To improve sensitivity to detection of the earliest biological damage caused by Alzheimer pathology, researchers have suggested that neuropsychological assessment should focus on those aspects of cognition that have focal associations with the regions of earliest neurodegeneration in AD (Marra et al., 2021; Venneri et al., 2019; Venneri, Mitolo, & De Marco, 2016). There is accumulating evidence that semantic memory has close associations with mediotemporal structures including hippocampus, parahippocampus and perirhinal cortex (Venneri et al.,

2011; Venneri et al., 2008). Semantic memory refers to the psychological representation of word meaning and conceptual encyclopedic knowledge acquired across the lifespan. Semantic memory is an aspect of memory that toghether with episodic memory forms declarative memory. Both forms of memory have their main anatomical association in mediotemporal structures. Episodic memory involves processing during storage and retrieval of contextualised material and it mainly reflects the operation of the hippocampus. Semantic memory, in contrast, involves processing during storage and retrieval of context free material and it mainly reflects the operation of perirhinal cortex (Mishkin, Suzuki, Gadian, & Vargha-Khadem, 1997). Semantic memory, differently from episodic memory, is not influenced by physiological age-related decline and, therefore, represents an aspect of neuropsychological decline to focus on when suspecting cognitive decline due to Alzheimer's disease. Furthermore, its anatomical association with entorhinal and perirhinal cortex is of particular interest since these anatomical structures are the earliest loci in which the first NFT deposits are detected in the earliest stages of AD development (Braak & Braak, 1991). Both these factors make semantic memory an ideal neuropsychological marker of the earliest signs of AD pathology enchroachment in the brain, at a stage when no evidence of deficits in episodic memory is yet detectable (Amieva et al., 2008). In addition, accumulating evidence indicates that the role of semantic memory is crucial not only for early detection, but it appears to be of great value in prognosis. There is evidence that MCI individuals who have episodic memory dysfunction coupled with a semantic memory deficit are more likely to progress to AD dementia than those who do not (Gustavson et al., 2020). There is also mounting evidence from longitudinal studies

that a less efficient semantic ability detectable a few decades before the onset of cognitive decline has predictive value of future AD-related dementia (Amieva et al., 2008; Vonk, Twait, Scholten, & Geerlings, 2020). A recent study also showed the predictive value of semantic performance in biomarker positive individuals (Papp, Rentz, Orlovsky, Sperling, & Mormino, 2017). This study showed that the coupling of amyloid positivity and poor semantic performance was a better predictor of future decline than biomarker positivity alone.

In neuropsychological assessment, semantic memory is tested relying on specific language mediated tests such as tests of category fluency, tests of letter fluency, of confrontation naming and of semantic association. Among these tests, the available evidence suggests that the best performing tests are those of category fluency (Venneri, Jahn-Carta, de Marco, Quaranta, & Marra, 2018).

As mentioned above, in the prodromal and established clinical dementia phases of AD, the most commonly observed symptom is that of an episodic memory failure. Episodic memory refers to that form of memory that allows people to re-live past experience with a specific contextual flavour. Deficits in this type of memory are a primary characteristic of patients with the amnestic form of MCI due to AD (Albert et al., 2011) and indicate the presence of hippocampal impairment. In neuropsychological assessment, a deficit in episodic memory is detected with tests measuring delayed recall of verbal and non verbal material such as tests of prose recall or delayed copy of complex figures. These tests are not only of diagnostic value, but at the MCI stage also have excellent power to predict whom in this population will progress to established AD dementia (Gainotti, Quaranta, Vita, & Marra, 2014).

Other cognitive abilities such as executive functions and visuospatial processing skills are affected by the deleterious effect of AD pathology at later stages of the disease. The only exceptions to this standard neuropsychological profile are those observed in rare forms of AD that manifest as posterior cortical atrophy (Caine, 2004) or other atypical variants of AD, such as the logopenic form (Henry & Gorno-Tempini, 2010) or the frontal form (Johnson et al., 1999).

2.3 The neuroimaging profile of AD

In recent years, neuroimaging has greatly added to progress by contributing to an understanding of the basic mechanisms of AD. Various neuroimaging methods have allowed the characterisation of different pathophysiological aspects of this disease, namely changes in structure and morphology, detection of specific markers involving radiotracers using techniques of molecular imaging, alterations of cerebral blood flow and brain metabolism that all can contribute to ealier detection and improved clinical diagnosis of AD. Despite their crucial contribution, clinically, evidence from routine neuroimaging plays a secondary role in AD diagnosis and prognosis in daily medical practice. This is primarily because clinical application of the most sophisticated imaging techniques lags behind their research use and the current contribution of neuroimaging to clinical diagnosis is still based on a mere qualitative evaluation limited to simple visual inspection of structure, cerebral blood flow and more rarely brain metabolism scans. Molecular imaging is only used for research purposes, for selecting people for inclusion in clinical trials, and only in some rare instances is this form of imaging approved for clinical use.

Evidence shows that magnetic resonance imaging (MRI) is the technique that provides the greatest and most important contribution to AD diagnosis. There is, however, a large number of people who experience cognitive symptoms and who do not have access to MRI imaging. Most people with suspected cognitive decline are often only offered a Computerised Tomography (CT) brain scan that provides limited contribution to diagnosis by excluding alternative gross causes of cognitive decline (Pasi, Poggesi, & Pantoni, 2011). MRI, especially T1 weighted (T1W) structural scans can detect regional atrophy, mainly atrophy of mediotemporal regions and of the hippocampus. It is possible, when MRI scans are acquired at high field and as high resolution images, to have a quantitative measure of global and regional volume loss, and even of volume loss in specific hippocampal subfields, providing crucial evidence of early neuronal loss in these regions (Zheng et al., 2018). Atrophy in these regions has a positive correlation with NFT load, but correlates negatively with cognitive performance, indicating that it represents a good proxy of the early clinical manifestations of AD. Significant volume loss in regions of the hippocampus is detected quite early at the MCI stage and grossly detectable atrophy in this region, as well as more globally througout the cortex, is even more noticeable in patients with established AD dementia (Frisoni, Fox, Jack, Scheltens, & Thompson, 2010).

Nuclear medicine techiques also have clinical applications, but they are not always equally sensitive or specific. One of the most clinically used technique is single photon emission computerised tomography (SPECT). SPECT scanning provides information about the brain regional blood flow. Its typical read-out in AD is that of a patten of hypoperfusion emcompassing precuneus, posterior cingulate, portions

of the lateral parietal lobes, mediotemporal and association temporal cortex bilaterally (Wollman & Prohovnik, 2003; Yeo, Lim, Khan, & Pal, 2013). This pattern is already detectable in the prodromal mild cognitive impairment stage of AD (Devanand et al., 2010; Huang, Wahlund, Svensson, Winblad, & Julin, 2002; Matsuda, 2007). With progression of disease, hypoperfusion of the hippocampus, fusiform girus and more widely the whole temporal lobe bilaterally becomes more pronounced {Kogure, 2000 #468}. For decades, acquisition of brain SPECT scans as a diagnostic aid was the norm in clinical pratice, but more recently evidence has accumalated suggesting poor specificity when used on its own (Davison & O'Brien, 2014; Yeo et al., 2013), leading to a significant reduction of its use in clinical practice in the diagnostic pathway of early AD.

Imaging techniques such as 18-fluorodeoxyglucose positron emission tomography (18-FDG PET) that assesses brain metabolism by measuring glucose uptake by neurones have, instead, gained in popularity, especially in research contexts, while their clinical use is more limited to specialised settings. There are several studies that suggest that measures of brain metabolisms are essential in detecting early changes and that these also have prognostic value (Caminiti et al., 2018; Pilotto et al., 2018). Indeed an 18-FDG PET scan returning a normal pattern of brain metabolism would rule out the presence of a potential neurogenerative pathology as the cause of any experienced cognitive difficulties. In contrast, an abnormal pattern of brain metabolism as typically seen in AD, with a reduced glucose uptake in mediotemporal, temporal, parietal regions and in posterior cingulate, all structures strongly affected by AD pathology, would have a sensitivity well above 90% (Marcus, Mena, & Subramaniam, 2014; Perani et al., 2014).

In more recent years, PET molecular imaging has become an essential technique for the early detection of AD pathology in the brain, although it remains largely a research tool. After the seminal work by Klunk et al (2004) using the 11-C Pittsburgh compound B (PIB), a tracer that binds to amyloid deposits in the brain, more practical (with a longer half-life) fluorine-based tracers have been produced and made available including florbetapir, florbetaben and flutemetamol [see (Yeo, Waddell, Khan, & Pal, 2015) for a review]. The pattern that imaging using these tracers detects in typical AD is that of Aβ deposits localised in parietal, temporal and in the frontal portion of a large scale functional brain network called default mode network (DMN) (Herholz & Ebmeier, 2011). Several tracers have also been produced (although only as research tools) that bind to tau deposits (Y. T. Wang & Edison, 2019). The most common pattern detected in AD is that of tau deposits localised in mediotemporal cortex and other limbic structures extending to temporal, parietal, frontal and occipatal cortex with increasing disease severity. Only one of these radiotracers, TAUVID^{TM} has been recently approved for clinical use by the Food and Drug Administration (FDA) in the USA (Jie, Treyer, Schibli, & Mu, 2021). The clinical availability of a tau tracer marks the reaching of a significant milestone in AD imaging research and it is a significant advancement as tau deposits are more aligned with clinical symptoms, making tau imaging a more realistically useful *in vivo* imaging biormarker.

Functional MRI (fMRI) imaging in resting state has also become of interest in the early detection of AD. The typical pattern of changes in spontaneous activity is that of progressively decreasing activity in the DMN, a large scale functional brain system in which the posterior cingulate cortex is said to have a central coordinating

role. The DMN encompasses many brain regions that are the focus of progressive amyloid depositions as well as of progressive atrophy (Buckner, Andrews-Hanna, & Schacter, 2008; Greicius, Srivastava, Reiss, & Menon, 2004). AD neurodegeneration appears to follow a pattern of progressive spreading across the topography of this network (Seeley, Crawford, Zhou, Miller, & Greicius, 2009), with the earliest reductions of activity beeing detected mainly in posterior regions focusing around the posterior cingulate cortex and precuneus (Jones et al., 2016; Jones et al., 2011; W. Koch et al., 2012).

Resting state fMRI can provide an overview of brain metabolism without the need for using any radioactive tracers (as required with PET). The versatility and advancement in image analysis methods, however, also allow the detection of insight about functional connectivity among brain areas. By studying the temporal correlation of neural activity among distinct brain areas it is possible to infer which areas are functionally connected (Friston, 2011; Friston, Frith, Liddle, & Frackowiak, 1993). The appropriate MRI acquisition to obtain this type of information is the echoplanar imaging technique that measures the *Blood Oxygenation Level Dependent* (BOLD) signal that exploits the measurement of varying levels of oxygenated haemoglobin to infer the level of metabolic activity of specific brain areas. By analysing the BOLD signal at rest, it is possible to obtain information about the brain spontaneous activity and inter-regional communication (Raichle & Mintun, 2006), but without the inter-individual variability that would be present when the BOLD signal is measured in response to a task. This method is particularly useful in AD, since performing a task in the scanner is often very demanding and difficult for patients with cognitive decline. Two distinct analytical

methodologies can be used to study spontaneous neural activity and functional connectivity at rest: a region of interest (ROI) approach and a whole brain independent component analysis (ICA) approach. An ROI approach requires an *a priori* selection of brain regions from which the BOLD signal is extracted. By performing correlations between the extracted signal of the selected ROIs or between a sigle ROI and the whole brain it is possible to assess their signal temporal correlation and infer how functionally connected these brain regions are (J. Wang et al., 2009). Conversely, the ICA method is based on the assumption that neural activity comes from independent sources of signal and that the observed activity is the combination of signal from these sources. Its aim, therefore, is that of detecting and separating these independent sources of signal, also known as brain functional networks (Rosazza, Minati, Ghielmetti, Mandelli, & Bruzzone, 2012). In the course of AD, the regions of one of these brain functional networks, the DMN, especially the posterior cingulate, precuneus, lateral temporal and parietal cortex, hippocampus and medial frontal cortex, are particularly vulnerable to the effects of AD pathology as shown by studies in which parallels have been drawn with target sites for progressive Aβ accumulation and atrophy (Hedden et al., 2009). This network has an important role in efficient cognitive performance and has to deactivate when people actively engage in a cognitive task. In addition, the regions part of the DMN sustain basal brain activity when at rest (Raichle et al., 2001), and support memory consolidation, mentalising, mental imagery and a range of other cognitive abilities (Fox & Raichle, 2007). As well as observing abnormalities in the DMN, a progressive functional isolation and disconnection of the hippocampus has also been detected in patients with AD (Allen et al., 2007; L. Wang et al., 2006).

These are pathological phenomena that are crucial in triggering the clinical manifestation of AD symptoms. In addition, reductions in connectivity between the posterior cingulate and precuneus, regions of the posterior part of the DMN, and the hippocampus are also key features of AD (Greicius et al., 2004). This pattern of findings is supported by evidence from PET and SPECT studies that have reported significant reduction in uptake of radiotracers in posterior cingulate, precuneus, hippocampus and other neighbouring regions within the DMN (Bradley et al., 2002; Minoshima et al., 1997).

The published studies suggest that abnormalities in regions of the DMN are already detectable at the MCI stage. Indeed, a more limited deactivation of regions in the DMN while engaging in a cognitive task has been observed in patients with MCI, and deactivation is even more limited at the dementia stage of AD. This more limited deactivation ability is noted in the early phase of the process of deactivation indicating reduced reactivity and a more limited adaptation of neural activity in the DMN (Rombouts, Barkhof, Goekoop, Stam, & Scheltens, 2005). Reduced neural activity in regions of the DMN is also predictive of future progression to AD dementia in MCI (Dennis & Thompson, 2014; Rombouts et al., 2005; K. Wang et al., 2007). In summary, the acculating body of evidence suggests that the regions of the DMN progressive reduction in neural activity reflect the parallel progression of neuropathology (Buckley et al., 2017). This suggestion is supported by a similar pattern of reduction in DMN neural activity detectable by resting state fMRI studies of healthy carriers of the *APOE* ε4 allele (Filippini et al., 2009) or in carriers of mutations (*PSEN1* and *PSEN2*) responsible for the autosomal dominant form of familial AD (Chhatwal et al., 2013).

In addition to alterations in the DMN, dysruption of neural activity is also observed in another large scale brain functional network, the salience network. This latter network involves the anterior portion of the insular cortex, the dorsal part of the anterior cingulate cortex, the amygdala and the striatum. This network has as its main function that of detecting personal and extra-personal relevant salient stimuli with the aim of guiding one's behaviour (Seeley et al., 2007). The salience network is linked by a dynamic relationship to the DMN that would guide shifting of attention, access to cognitive resources for general cognitive processing as well as for specific cognitive domains. (Menon & Uddin, 2010). In AD, neural activity in the DMN and in the salience network are said to be anticorrelated, with progressive reduction of neural activity in DMN being accompanied by a progressive increase in neural activity in regions of the salience network (Zhou et al., 2010).

2.4 Cerebral spinal fluid and blood biomarkers

The quantification of Aβ42, total tau and phosphorylated tau in the cerebral spinal fluid (CSF) is the most reliable way to detect the presence of Alzheimer's disease *in vivo*. Decreases in the concentration of Aβ42 in the CSF reflect the presence of amyloidosis, and of abnormal deposition and faulty clearance of the amyloid protein in the brain, therefore flagging, overall, the pathophysiological processes that characterise AD. An increase in the concentration of total tau in the CSF reflects the process of neurodegeneration and cortical neuronal loss, while a CSF increase in phosphorylated tau signifies an increase in the accumulation of neurofibrillary tangles (Seppala et al., 2012).

All together, these three biomarkers achieve a good diagnostic accuracy with a sensitivity and specificity of 85-90% in detecting AD at the dementia stage or even at the prodromal mild cognitive impairment stage. In this latter case, their utility is greater in terms of negative predictive value, since concentrations of CSF Aβ42, total tau and phosphorylated tau in the physiological range, exclude a diagnosis of AD (Olsson et al., 2016).

CSF biomarkers have increased in use more recently in research settings, since they have been included as important factors in the selection of patients for research studies or clinical trials (J. Cummings, 2020), although in this latter case amyloid imaging has become of more frequent use than CSF biomarkers. Their use has also increased due to improved methodologies, increased reproducibilities and more standardised cut-offs (J. H. Kim et al., 2017; Oudart et al., 2020). Their use in common clinical practice and, more generally, in clinical research of observational nature has not taken off, however, since their invasive nature, given that they require a lumbar puncture, does not make them popular with patients who are often not keen to undergo this procedure, making their wide scale applicability for screening impractical.

In more recent years, considerable research efforts have been made to develop blood based technology that can equally successfully detect alterations in levels of Aβ and neuronal injury (Zetterberg, 2019). Blood-based assays represent a considerable challenge for disease of the central nervous systems. Proxies of amyloidosis and neuronal damage need to cross the blood brain barrier to be detectable in blood assays. More recent evidence suggests that, despite technical challenges, it is now possible to detect levels of the Aβ42/Aβ40 ratio in the blood

indicative of cerebral amyloidosis with an accuracy of 70-90% that very nearly approximate the accuracy of CSF based assays. It is also possible to detect markers of neurodegeneration in the form of neurofilament light in serum or plasma, reflective of underlying neuronal injury (Ashton et al., 2019; Bangen et al., 2021; Darmanthe, Tabatabaei-Jafari, Cherbuin, & Alzheimer's Disease Neuroimaging, 2021). Finally, sophisticated immunassays can also detect concentrations of phosphorylated tau in the plasma that strongly correlate with the concetration of phosphorylated tau detected in CSF (Chong et al., 2021).

Progress in this direction is of great clinical importance, because the availability of reliable blood-based biomarkers of AD would make large scale screening for this disease a real prospect, easily scalable to a large number of the adult population in the at risk age range.

2.5 Issues with differential diagnosis

Early and differential diagnosis of AD is a complex clinical task not only because symptoms (Backman, 2008) and pathological features (Neuropathology Group. Medical Research Council Cognitive & Aging, 2001; Wyss-Coray, 2016) may overlap with the physiological effects of ageing on the brain, but also because the early clinical phenotype and underlying pathology show significant overlap with other neurodegenerative conditions (Kalaria et al., 2001; Karantzoulis & Galvin, 2011; Stephan et al., 2012). Indeed, subtle memory decline characterises individuals' experience of ageing cognition (Buckner, 2004; Nyberg, Lovden, Riklund, Lindenberger, & Backman, 2012), while decline in linguistic and executive abilties as well as alterations of behaviour can be observed both in AD,

especially in its frontal variant, and in decline caused by frontotemporal degeneration (Padovani et al., 2013; Reul, Lohmann, Wiendl, Duning, & Johnen, 2017). Similarly visuospatial and visuoperceptual decline can characterise early AD, especially in its posterior variant, as well as being a core feature of dementia with Levy bodies, with similarities also being observed in neuroimaging markers (e.g. hypometabilism in posterior regions) (McMonagle, Deering, Berliner, & Kertesz, 2006; Oda, Yamamoto, & Maeda, 2009; Whitwell et al., 2017). It is, therefore, of primary importance to intensify research efforts to improve methods that can lead to a more accurate early and differential diagnosis of AD.

3. Treatment

Despite extensive research effort and large investments by the pharmaceutical industry, there is still no cure for Alzheimer's disease. The only form of treatment that is currently approved is symptomatic, acting on neurotransmission to improve synaptic and neuronal function. The available treatment has remained the same for the last 25 years. The present forms of treatment have been shown to have a modest effect on delaying clinical progression and some time-limited effect on improving cognition in patients with mild to moderate AD (J. Birks et al., 2000; J. S. Birks et al., 2000; Blesa et al., 2003). The basic mechanism of action is that of improving cholinergic transmission by inhibiting the cholinesterase enzyme. These drugs are the outcome of the evidence of a clear cholinergic deficit and deterioration of cholinergic nuclei in AD (Giacobini, 2003, 2000). In addition to cholinergic based treatment, there is one additional approved form of treatment for the moderate to severe stages of AD. This latter acts on the glutamatergic system and is a non competitive antagonist of N-methyl-D-aspartate (NMDA) receptors (Reisberg et

al., 2003). It is used normally as an *add-on* treatment in combination with cholinesterase inhibitors. Cholinergic and glutamatergic forms of treatment have been explored given the role of these neurotransmitters in memory processes, since memory complaints are the main symptoms experienced by patients when the disease becomes clinically manifest. Indeed, most of the areas associated with memory processes in mediotemporal lobe have a primarily cholinergic innervation. In addition, blockage of NMDA receptors optimises neural transmission and facilitates the reduction of neurotoxicity resulting from the influx of Ca^2 ions in the intracellular environment caused by glutammate (Reisberg et al., 2003). The combination of these effects manifests as modest, but time-limited, cognitive improvements and better performance in every day life activities.

In the last decade, several attempts have been made to slow down the progression of the disease by trying to limit its neuropathological progression. Treatments based on the use of monoclonal antibodies that target Aβ, and even more recently tau, have been the object of a plethora of clinical trials. However, although most clinical trials have shown that these so called disease modifying treatments are successful in removing their respective abnormal deposits from the brain, no clinical benefits have been detected, and none so far has been shown to modify the course of the disease (J. Cummings, 2018; J. Cummings, Ritter, & Zhong, 2018; Lahiri, 2019; Schott, Aisen, Cummings, Howard, & Fox, 2019), even when treatment has been provided years before onset of symptoms as shown in trials of asymptomatic individuals at risk for the genetic form of the disease (Salloway et al., 2021). The original enthusiam towards this class of treatment has, therefore, progressively waned (J. Cummings, 2018; J. Cummings et al., 2018). Recently,

however, provisional emergency approval has been granted in the USA to one form of these treatments, aducanemab, that has removal of amyloid plaques from the brain as its target (https://www.fda.gov/news-events/press-announcements/fda-grants-accelerated-approval-alzheimers-drug). The approval of aducanemab has been given even though no evidence of clinical benefit had been found initially in the two phase 3 clinical trials of this drug (EMERGE and ENGAGE) that were halted early following the negative outcome of a futility analysis of interim data that indicated that it were unlikely that any improvements would be detected in any of their primary outcome measures. A subgroup analysis in patients who had received a high dose was subsequently carried out and the findings appeared to indicate a clinical benefit (von Hehn & Tian, 2019), However, alternative explanations were offered for this finding, with some authors suggesting that divergence in progression of patients in the placebo arm might have resulted in this significant finding (Knopman, Jones, & Greicius, 2021). Despite the controversy over its efficacy data, for the first time for a drug targeting the nervous system, aducanemab received approval by the USA regulatory authority on the basis of evidence of target engagement and successful removal of amyloid plaques. A recent article has reported further analyses of the entirity of data of two phase 3 clinical trials. These analyses of the data of the two halted trials were justified by the assertion that, although previous futility analysis had met the prespecified futility criteria, additional evidence had emerged that indicated that there was violation of two assumptions on which the futility analysis was based. These analyses showed that in one of the two trials, EMERGE, significant change from baseline after 78 weeks of treatment was obtained in the primary outcome measure

selected in this study, i.e. the Clinical Dementia Rating Sum of Boxes (CDR-SB), while this was not the case, however, for the ENGAGE trial (Budd Haeberlein et al., 2022). Despite publication of a positive finding in one of these two phase 3 clinical trials, controversy remains on the actual clinical benefit of this treatment since two clinical trials with identical design have yielded different results. It is not clear, in fact, on which basis the positive findings of one trial should outweight the negative findings of the other (Knopman et al., 2021). Furthermore, approval for aducanemab was granted for AD without any specification of disease stage, even if trials conducted so far were all with people in prodromal to mild stages of AD. This regulatory decision sparked outrage in the clinical and scientific communities, leading the American regulators to limit the use of this drug to early disease stages only a few weeks after the original decision. Although, approval of this drug has, in contrast, received an enthusiastic welcome from patient organisations and some of the hard supporters of the amyloid hypothesis of AD, the consensus in the clinical and scientific community worldwide is that the road to an effective form of disease modyfing treatment for AD is still long and the prospect of a cure remains still distant.

4. The present work

It is clear from the overview presented above that there are numerous open questions in AD. The lack of a clear understanding of its pathological mechanisms and their interaction, the difficulties in clinical diagnosis and the limited availability of non invasive biomarkers are all making the quest of an effective form of treament or cure difficult. Progress is also hindered by the heterogeneity of clinical

presentation and the uncertainty surrounding the role in AD pathology of comorbidities (e.g. vascular pathology), genetic risk and multiple pathological processes.

The work presented in this thesis attempts to address some of the issues that remain open or controvertial in AD. There is a consensous of views among researchers that progress is hindered by accuracy of diagnosis of patients included in clinical trials and by the late stage of diagnosis. The publications included in this thesis focus on addressing these points, namely; how to improve accuracy of clinical diagnosis in prodromal stage with the help of artificial intelligence alogrithms; and how to refine markers by selecting those features that are not influenced by the process of normal ageing and represent specific and early proxies of incipient disease. The main research questions that have been addressed, therefore, are as follows:

1. Can a multidomain approach that combines cognitive and multimodal imaging variables with classificatory algorithms using artificial intelligence methods be more useful than current clinical practice in detecting patients in the prodromal stage of AD?
2. Are there any distinctive cognitive or neuroimaging markers that distinguish age related from disease related decline and are these detectable early in the disease course?

One publication is presented to addresses the first question.

De Marco M, Beltrachini L, Biancardi A, Frangi AF, Venneri A. Machine-learning Support to Individual Diagnosis of Mild Cognitive Impairment Using Multimodal MRI and Cognitive Assessments. Alzheimer Dis Assoc Disord. 2017 Oct-Dec;31:278-286. doi: 10.1097/WAD.0000000000000208.

In this article machine learning algorithms were used to assess the classificatory power of a combination of cognitive and structural imaging features, reflecting data collected in current clinical practice in neurology based memory services, and compare it with the classificatory power of a combination of cognitive, structural imaging and functional imaging features for the individual classification of people at the prodromal stage of AD. The new approach, combining cognitive and multimodal imaging features revealed a superior classificatory power at the individual level, reaching a very accurate level of classification. The findings suggest that, at this early stage of neurodegeneration, regional metabolic dysfunction is dectectable and it is a better indicator of disease when combined with cognitive and regional atrophy data than regional atrophy and cognitive deficits alone.

Four publications are presented that address the second question.

De Marco M, Venneri A. Volume and connectivity of the Ventral Tegmental Area are linked to neurocognitive signatures of Alzheimer's disease in humans. Journal of Alzheimer Disease, 2018 doi: 10.3233/JAD-171018

This study has been the first to translate in humans findings detected in a mouse model of AD (Nobili et al., 2017). In this study Nobili et al, using a mouse model of AD, found that a small brainstem nucleus, the Ventral Tegmental Area (VTA), shows substantial loss of dopaminergic neurons well in advance of the appearance of amyloid plaques. Loss of this type of neurons in this region affects the number of afferent dopaminergic connections to the hippocampus, leading to reduced plasticity of neurons located in the CA1 area of the hippocampus and worsening of cognitive performance. Cognitive performance, however, could be improved by administration of treatment with selegiline, an L-DOPA compound. The evidence from this study gave support to earlier claims that impairment of dopaminergic pathways is a very early manifestation of AD and that it plays a causative role in AD (Nobili et al., 2017). Based on this animal study, our study translated the findings in humans. Using structural neuroimaging data acquired with high resolution MRI, our study demonstrated how volumetric measures of the VTA correlate with volumes in the hippocampus and performance on memory tests in older adults. We were also able to demonstrate that hippocampal volume and memory performance were influnced by the level of functional connectivity between the VTA and the hippocampus (De Marco & Venneri, 2018). These findings highlighted the need for further clarification of the role of VTA degeneration in AD aetiopathogenesis and its potential for therapeutic interventions in the earliest phase of this disease. The following study was designed to clarify whether dopaminergic loss in the VTA preceeds damage in other brainstem nuclei and whether it is a good predictor of future clinical manifestations.

Venneri A, De Marco M. Reduced monoaminergic nuclei MRI signal detectable in pre-symptomatic older adults with future memory decline. Scientific Reports 2020 10 (1), 1-11

Because the research question addressed in this study required a period of observation of several years, it would not have been possible to produce timely evidence with data collected in house. For the purpose of this study, data from the freely available Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu) were used. The ADNI database contains data (clinical, multimodality neuroimaging, cognitive and neuropsychiatric assessments) collected longitudinally from healthy elderly, people with mild cognitive impairment and people with dementia of the AD type. It is a multicentre initiative based in the United States that has the aim to monitor progression of disease and to discover potentially clinically useful markers of disease. The datasets used in this study were those from healthy volunteers for whom follow up assessments highlighted the presence of cognitive decline compatible with an AD aetiology. These datasets were from people who have been monitored longitudinally with full assessments for several years and represented the perfect opportunity to test the validity of our earlier findings. The results were consistent with our earlier discovery and suggested that VTA degeneration preceeds degeneration in any other brainstem nucleus. Furthermore, declinining participants had lower levels of amyloid detected in cerebral spinal fluid, but no difference in total tau or phorylated tau levels was present. This study provided compelling evidence that,

indeed, VTA degeneration is an early event in the AD aetiopathogenesis. We were not able to ascertain whether VTA degeneration preceeds amyloid deposition, whether degeneration in VTA is linked to a pathological process fostered by levels of soluble amyloid or whether the two disease mechanisms are completely independent. Additional work is needed to address these issues.

Venneri A, Mitolo M, Beltrachini L, Varma S, Della Pieta' C, Jahn-Carta C, Frangi AF, De Marco M. Beyond episodic memory: Semantic processing as independent predictor of hippocampal/perirhinal volume in aging and Mild Cognitive Impairment due to Alzheimer's Disease. Neuropsychology, 2019 Feb 18. doi: 10.1037/neu0000534.

Our earlier machine learning study had highlighted that the features with the highest classificatory power were those of semantic memory and functional activity in parts of the temporal cortex. This study was designed to gain a better understanding of the reasons why semantic memory had been revealed as an excellent classifiers of people in the prodromal stage of AD. A significant association was found with parts of the hippocampus (especially on the left) and the perirhinal cortex suggesting that measures of semantic free recall, such those derived from category fluency tests, represent good and specific proxies of regional neurodegeneration and, given their non invasive and quick mode of administration, could be used for preliminary general early screening in that population age range at risk of developing AD related cognitive decline and AD dementia.

While methods of early detection are important to implement effective preventative or limiting strategies to delay cognitive decline, or to improve accuracy of diagnosis in people enrolled in clinical trials, soon there will be an unmet clinical need. In fact, the provisional approval of the first disease modifying treatment for AD will highlight the need for more specific and easy to use markers of disease that have to be sensitive to the earliest pathological changes caused by AD. Even if blood based disease biormarkers are increasingly becoming more reliable, it is also the case that diagnosis cannot be devoided of clinical manifestations. Indeed recent guidelines reccommend that a diagnosis of AD cannot be based only on biological evidence, but needs to be coupled with the presence of some clinical symptoms. (Dubois et al., 2021). These cannot be those typical of the clinical phase of AD, i.e. deficits in episodic memory and new learning, but there needs to be more subtle evidence of decline of cognitive functioning, not confounded by ageing effects and relatively specific to the early regional degeneration that occurs silently in the preclinical stage of the disease.

De Marco M, Ourselin S, Venneri A. Age and hippocampal volume predict distinct parts of default mode network activity. Scientific Reports, 2019 Nov 5;9(1):16075. doi: 10.1038/s41598-019-52488-9.

As a parallel to the previous study, the final article included in this thesis reports a study that was conceived to clarify the role of regional brain activity that had emerged as a crucial classifier to reach a very high level of accurate individual classification in the machine learning study presented earlier in this thesis. From

previous studies, it was expected that the regions that should have contributed to classification would have clustered in the areas that are part of the posterior default mode network (Buckner et al., 2008; Greicius et al., 2004; Jones et al., 2011). It was especially expected that activity detected in posterior cingulate and precuneus regions should have been the ones with the highest classificatory power (Anchisi et al., 2005; Perani et al., 2014). However, the regions with the highest level of classificatory power were those in posterior temporal cortex. This interesting but unespected finding led to the question of whether the findings of crossectional studies of the DMN in mild cognitive impairment might have been confounded by the effect of the process of physiological ageing in addition to the regional effects of disease. This study, therefore, was designed to test to which exent the repeated finding of posterior cingulate decline in activity is a specific outcome of progression of AD pathology deposition in those regions or an ageing associated epiphenomenon.

The findings of this study showed that both ageing and AD had a strong association with the level of functional activity in the posterior cingulate/retrosplenial cortex and precuneus. More in depth analyses showed that there was a strong statistical association beetwen age (expressed as days of life) and posterior DMN expression in these regions. This, however, was not the case for a disease proxy (expressed by hippocampal volume). Hippocampal volume (used as an indicator of severity of disease related neurodegeneration) was instead a predictor of functional activity in regions of the temporal parietal junction, a finding that strongly confirmed the result of the earlier machine learning study presented in this thesis. These results, therefore, suggest that postero-medial DMN down-regulation may not be a specific

byproduct of neurodegenerative processes, but might probably highlight regional brain vulnerability to degeneration. Down-regulation of activity within structures in the temporal parietal junction region, instead, was found specifically associated with volumetric indices of the hippocampus and may be a reflection of early-stage regional accumulation of pathology. Taken together with our previous findings, the results of this study suggest tha measures of functional activity in association cortex within the temporal lobe as well as other regions within the temporal parietal junction territory might be useful in clinical practice to detect early indicators of abnormal ageing.

5. General conclusions

Alzheimer's disease aetiopathogenesis (in its sporadic occurrence) remains largely unexplained despite significant progress. Indeed, we have a clearer understanding of pathological and clinical manifestations. Despite progress, the subtle and insidious onset, the confounding effects of ageing and the relative lack of specificity of its early clinical manifestations have slowed down progress in finding effective diagnostic methods, in identifying specific markers not only of the biological presence of possible pathological processes in the brain, but capable of revealing subtle specific symptoms. In turn, all of the above has hampered the development of an effective cure. The evidence presented in this thesis contributes to a better understanding of those early indicators that might be clinically meaningful and might have diagnostic and prognostic value.

The findings reported in the first article included in this thesis clearly show that it is possible to improve on accuracy of individual clinical diagnosis by using

extensive cognitive testing and multimodal imaging coupled with machine learning algorithms. Methods borrowed from artificial intelligence have entered modern medicine and even in the field of AD previous research has encouraged this approach as a diagnostic aid (Antila et al., 2013). However, previous work focused on structural imaging and limited cognitive measures, basically using standard investigations adopted in current clinical practice in combination with artificial intelligence. The classificatory power of this approach, however, is better than accuracy of clinician based diagnosis as shown in our previous work (Beltrachini et al., 2015) and in the article included in this thesis, but the novel inclusion of measures of region to region functional connectivity, structural volumetric features and cognitive features in the classificatory algorithm represents a considerable improvement on current clinical practice with the combination of these features reaching 95% accurate individual classification. This study also hightlighted a series of important findings that acted as the catalyst for the other studies included in this thesis. At the cognitive level, it was clear that a recurrent finding was that category fluency emerged as a strong classifier in all models. In contrast, hippocampal volume did not emerge as a constant strong feature in the individual discrimination of MCI from healthy controls and, similarly, functional connectivity of the posterior cingulate region did not appear among the top classifiers. This evidence prompted the additional studies included in this thesis.

Earlier longitudinal population studies (increasingly supported by more recent evidence) had already given an indication that the best predictors of future dementia were tests of semantic memory (e.g. (Amieva et al., 2008; Vonk et al.,

2020)). Evidence had also been produced that when added to a prediction algorithm, semantic memory tasks would increase the accuracy of prediction of patients who developed AD dementia later in life (Papp et al., 2017). The novelty of our work is that we have demonstrated that semantic memory tasks are good proxies of selective neurodegeneration in the hippocampus and when added into a classificatory tool, the algorithm is more powerful and can improve individual classification of people with mild cognitive impairment reaching accurate classification in about 90% of cases.

Our work was also able to demonstrate that the addition of measures of functional activity in temporal association cortex to a classifying algorithm can boost accuracy of classification further to reach about 95%. Taken all together these findings show a significant improvement on current clinical practice, where accuracy at best is at about 70-75% if patients are thoroughly assessed with clinical assessment, extensive neuropsychological testing and structural imaging (Beach, Monsell, Phillips, & Kukull, 2012). However, the functional connectivity feature ranking as the highest classifier was not in the region of the posterior cingulate. Accuracy of clinical diagnosis is often hindered by the parallel effects of ageing or of alternative pathological processes that might confound the presenting phenotype. Furthermore, age related comorbidities also contribute to the severity of the presenting symptoms, to the detriment of accuracy of diagnosis. Despite extensive research that indicates down-regulation of brain activity in the posterior cingulate and precuneus, these regions were not among the features that improved accuracy of individual classification in mild cognitive impairment. This finding does not indicate that dysfunction in these regions does

not play a role in clinical progression, but rather that it is the outcome of the synergistic effect of ageing and disease related mechanisms and is not a specific outcome of AD. When tested specifically, our findings revealed that most of the variance in activity in the posterior cingulate regions and precuneus was indeed explained by variance in age and only a small cluster appeared to reflect severity of disease. This finding suggests that absolute levels of activity in specific regions are less useful when trying to identify a diseased pattern, but measures of region to region functional connectivity (especially between temporal and midline parietal structures) have more clinical utility and when added with measures of regional atrophy and scores on measures of episodic and especially semantic memory appear to lead to accurate discrimination between mild cognitive impairment due to AD and healthy ageing. The evidence from these studies is in line with neuropathological evidence that clearly suggests that brain multi-morbidity is often detected and common coexistence of AD, vascular, Lewy body and TDP 43 pathologies is frequent, particularly in the oldest part of the population (Kovacs, 2019; J. L. Robinson et al., 2018). The available evidence, therefore, indicates that the clinical phenotype is often confounded by the mixture of multimorbidity and ageing.

The final articles included in this thesis tried to address whether there are structural changes that might preceed hippocampal atrophy and that might be detectable at the preclinical stage of the disease. Indeed hippocampal volume did not appear to be of high discriminatory power in our earlier study, adding no additional classificatory power to that already given by cognitive tests and, when used on its own, reached levels of classification of about 70-75%, levels that are

not very different from clinical impression. The two articles included in this thesis, by translating into humans and extending the findings of animal work were able to demonstrate an early and exclusive degeneration of a brainstem dopaminergic nucleus, the ventral tegmental area, in those healthy individuals that later developed mild cognitive impairment. This early degeneration was present in parallel with presence of amyloidosis in these individuals. What these findings suggest, however, is that, while the presence of amyloidosis *per se* has no clinical significance, degeneration of the ventral tegmental area does, since volume loss in the VTA correlates with typical signatures of clinical AD, i.e. hippocampal volume and memory performance and, additionally, functional connectivity from the VTA explains both hippocampal volume and memory performance.

This finding suggests that VTA degeneration is a more direct proxy and predictor of future disease than amyloidosis is, since it is associated with level of cognitive performance, while amyloidosis is not. In addition to its early predicitive value, the dopaminergic dysfunction related to VAT degeneration might lead to a potential form of early treatment. Indeed, a recent clinical trial seemed to suggest that there is a degree of clinical benefit and therapeutic value in using dopaminergic augmentation in AD (G. Koch et al., 2020). Additional research is, however, needed to establish whether these clinical benefits and therapeutic value extend to the preclinical and prodromal stages of AD.

6. References

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7. Appendix 1

Ethical approval

Health Research Authority

NRES Committee Yorkshire & The Humber - Sheffield

Yorkshire and the Humber REC Office
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28 December 2012

Professor Annalena Venneri
Professor of Clinical Translational Neuropsychology
University of Sheffield, Department of Neuroscience
Medical School, N Floor, Rm N130
Beech Hill Road
Royal Hallamshire Hospital, Sheffield
S102RX

Dear Professor Venneri

Study title:	**Assessment of age and disease related cognitive impairment in normal volunteers and people with degenerative and vascular brain disease and assessment of the potential neuroplastic effect of non pharmacological treatment**
REC reference:	**12/YH/0474**
IRAS project ID:	**84442**

Thank you for your letter of 19 December 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Rachel Bell, nrescommittee.yorkandhumber-sheffield@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		18 September 2012
GP/Consultant Information Sheets	1.0	18 September 2012
Investigator CV		
Participant Consent Form: Patient 1	2	19 December 2012
Participant Consent Form: Patient 2	2	19 December 2012
Participant Consent Form: Volunteer 1	2	19 December 2012
Participant Consent Form: Volunteer 2	2	19 December 2012
Participant Information Sheet: Patient 1	2	19 December 2012

Participant Information Sheet: Patient 2	2	19 December 2012
Participant Information Sheet: Volunteer 1	2	19 December 2012
Participant Information Sheet: Volunteer 2	2	19 December 2012
Protocol		
REC application		
Response to Request for Further Information		19 December 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/YH/0474 **Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

8. Appendix 2

Copyright clearance

DOI: 10.3233/JAD-171018
Citation: Journal of Alzheimer's Disease, vol. 63, no. 1, pp. 167-180, 2018

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9. Appendix 3

Publications

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Machine-learning Support to Individual Diagnosis of Mild Cognitive Impairment Using Multimodal MRI and Cognitive Assessments

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Background: Understanding whether the cognitive profile of a patient indicates mild cognitive impairment (MCI) or performance levels within normality is often a clinical challenge. The use of resting-state functional magnetic resonance imaging (RS-fMRI) and machine learning may represent valid aids in clinical settings for the identification of MCI patients.

Methods: Machine-learning models were computed to test the classificatory accuracy of cognitive, volumetric [structural magnetic resonance imaging (sMRI)] and blood oxygen level dependent-connectivity (extracted from RS-fMRI) features, in single-modality and mixed classifiers.

Results: The best and most significant classifier was the RS-fMRI +Cognitive mixed classifier (94% accuracy), whereas the worst performing was the sMRI classifier (~80%). The mixed global (sMRI+RS-fMRI+Cognitive) had a slightly lower accuracy (~90%), although not statistically different from the mixed RS-fMRI+Cognitive classifier. The most important cognitive features were indices of declarative memory and semantic processing. The crucial volumetric feature was the hippocampus. The RS-fMRI features selected by the algorithms were heavily based on the connectivity of mediotemporal, left temporal, and other neocortical regions.

Conclusion: Feature selection was profoundly driven by statistical independence. Some features showed no between-group differences, or showed a trend in either direction. This indicates that clinically relevant brain alterations typical of MCI might be subtle and not inferable from group analysis.

Key Words: machine learning, magnetic resonance imaging, semantics, hippocampus, resting-state

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Mild cognitive impairment (MCI) identifies adults who experience impairment in neuropsychological abilities, while retaining daily-life independence. The range of possible etiologies is heterogenous, with Alzheimer disease (AD) often being a prime suspect.[1] Nonpathologic processes of senescence, however, may also trigger a measurable decline in cognitive functioning,[2] and it is not uncommon that healthy adults complain of their declining cognitive abilities. This conceptual overlap is further complicated by additional factors. First, thresholds of impaired cognitive performance have been operationalized in many ways.[3] Second, variability in the choice of cognitive tests and their procedure of administration generates different diagnostic outputs.[4] Third, cross-cultural differences exist in test performance,[5] but this is rarely acknowledged. Fourth, raw neuropsychological scores may distribute skewly,[6] compromising the validity of the descriptors used to set the threshold of "normality". Fifth, high levels of education may mask the presence of cognitive impairment.[7]

Recently, revised versions of consensus guidelines have incorporated supporting evidence from neuromolecular imaging and cerebrospinal fluid biomarkers, for diagnosing MCI due to AD.[8] Despite the theoretical robustness of this approach, these techniques are not appropriate for characterizing AD burden in asymptomatic adults or patients with nonprogressive/nonpersistent MCI.[9] A more viable contribution is that of structural magnetic resonance imaging (sMRI) and resting-state functional magnetic resonance imaging (RS-fMRI). Both appear useful to describe patients diagnosed with clinically established AD,[10,11] and RS-fMRI in particular is increasingly receiving attention by researchers, as it seems to be sensitive to very early pathologic alterations.[12] Although significant reduction of regional functional connectivity in MCI has been reported in cross-sectional,[13] and longitudinal studies,[14] this evidence is the result of group-level inferential statistics, which is of limited utility for the clinical classification of single individuals. Multivariate and machine-learning techniques offer the opportunity to build data-driven classificatory models which can predict group membership of each participant based on MRI features. A number of recent studies have implemented these classificatory techniques to identify MCI patients using RS-fMRI as a single source of diagnostic information,[15–18] or in combination with sMRI.[19–21]

In this study we used machine-learning methods to carry out classifications of participants with a diagnosis of MCI based on features extracted from cognitive performance, sMRI, and RS-fMRI, with a series of single-type and mixed classifiers. No specific hypothesis was formulated in association with cognitive classifiers as the diagnostic status was heavily dependent on cognitive performance. We hypothesized that RS-fMRI-based classifiers would be superior to the

others (quantitative expectation), and that the selected features would yield important connection with neuropathologic models of abnormal aging (qualitative expectation). A major goal was to understand to what extent and in what way such methodology would be of aid in clinical settings.

METHODS

Participants

In total, 139 inhabitants of the Venetian lagoon, older than 50 years and still independent in their daily activities were considered for inclusion. Candidates were either outpatients referred to neurological examination by their general practitioner because of suspected cognitive decline, or adults willing to take part in research projects because of personal interest and/or subjective cognitive concerns. All underwent a comprehensive medical examination led by an experienced neurologist between May 2011 and November 2014. This was based on the anamnestic information, a neurological screening, a clinical MRI protocol (including diffusion-weighted, T1-weighted, T2-weighted, and Fluid Attenuation Inversion Recovery images) which was inspected by a senior neuroradiologist, and a battery of cognitive tests administered and interpreted by an experienced neuropsychologist. Upon application of exclusion criteria, participants were allocated to 1 of 2 diagnostic categories: healthy adult having no objective cognitive difficulties (control), or patient diagnosed with MCI (patient). Diagnoses of MCI were established by a consensus of opinions among clinicians and clinical follow-ups. Diagnostic exclusion criteria were as follows: a Mini Mental State Examination score <24, ongoing treatments (psychotropic medication, cholinesterase inhibitors, memantine, drugs for research purposes, or with toxic effects to internal organs); a significant disease at clinical level; history of transient ischemic attack; diagnosis of severe vascular pathology; baseline structural MRI revealing different diagnostic patterns from those expected in MCI; presence/diagnosis of uncontrolled seizures; peptic ulcer; cardiovascular disease; neuropathy with conduction difficulties; significant disabilities; proof of abnormal baseline levels of folates, vitamin B12, or thyroid-stimulating hormone. "Technical" exclusion criteria were as follows: >1 missing entry in the database of cognitive scores; presence of relevant signal artefacts or excessive in-scanner motion. On the basis of application of these criteria, 50 controls and 50 patients matched as closely as possible at a group level for age, education levels, and sex ratio were included. Demographic characteristics of the final sample are reported in Table 1. This study was approved by the Institutional Review Board of the IRCCS Fondazione Ospedale San Camillo (Venice, Italy), protocol number 11/09-version 2. Informed consent was obtained from all participants.

MRI and Cognitive Data Acquisition

The MRI protocol (1.5 T Philips Achieva), including structural and functional acquisitions, was completed in a single session. Participants were instructed to keep their eyes closed without falling asleep and remain as still as possible for the full duration of the examination. Turbo-field echo T1-weighted images were acquired with the following characteristics: voxel dimension 1.10×1.10×0.60 mm; Repetition Time 7.4 ms; Echo Time 3.4 ms; Field of View 250 mm; matrix size 256×256×124; flip angle 8 degrees.

TABLE 1. Demographic and Neuropsychological Characteristics (expressed as means and standard deviations in parentheses) of the Sample

Variables	Healthy	MCI	Group Difference	
Demographic Factor			$P\ U_{\text{Mann Whitney}}/\chi^2$	
Age (years)	69.54 (5.88)	73.86 (6.31)	<0.001	
Education (years)	10.94 (4.60)	10.70 (4.33)	0.840	
Sex (F/M)	31/19	25/25	0.227	
Neuropsychological Test			$P\ U_{\text{Mann Whitney}}$	$P\ F_{\text{Corrected}}$
Mini Mental State Examination	28.98 (1.32)	27.46 (1.92)	<0.001	<0.001
Raven Progressive Matrices	30.14 (4.62)	27.34 (5.77)	0.015	0.029
Digit Cancellation Test	53.52 (5.27)	48.16 (7.91)	<0.001	0.001
Stroop Test—time interference	23.70 (8.99)	35.77 (18.48)	<0.001	<0.001
Stroop Test—error interference	0.97 (2.81)	3.02 (5.79)	0.002	0.126
Letter Fluency Test	34.74 (12.81)	31.34 (11.08)	0.145	0.234
Category Fluency Test	41.36 (9.92)	30.18 (8.66)	<0.001	<0.001
Token Test	34.50 (1.79)	34.18 (1.90)	0.305	0.256
Similarities Test	20.80 (5.02)	19.78 (4.37)	0.175	0.467
Confrontational Naming Test	19.17 (1.46)	18.48 (1.72)	0.019	0.044
Digit Span Test—forward	6.08 (0.92)	5.74 (0.90)	0.026	0.026
Digit Span Test—backwards	4.30 (0.95)	3.72 (0.81)	0.002	0.001
Paired Associates Learning Test	13.36 (4.02)	9.54 (3.77)	<0.001	<0.001
Prose Memory Test—immediate recall	9.88 (3.68)	6.72 (3.64)	<0.001	<0.001
Prose Memory Test—delayed recall	13.10 (4.71)	7.32 (4.48)	<0.001	<0.001
Corsi Block Tapping Test	4.82 (0.87)	4.22 (0.79)	0.001	0.002
Visual Supraspan Test	20.70 (6.60)	13.20 (8.33)	<0.001	<0.001
Rey-Osterrieth Figure—copy	32.47 (3.65)	29.55 (6.18)	0.008	0.028
Rey-Osterrieth Figure—recall	15.98 (5.66)	8.45 (4.59)	<0.001	<0.001

Between-group differences in cognitive performance were analyzed both with Mann-Whitney tests as well as ANOVAs, correcting for age and years of education. A Bonferroni-corrected P threshold equal to 0.002 was adopted as the appropriate significance level. There were only 3 missing data points: 2 participants missing their Token Test score (1 control and 1 patient) and 1 participant (patient) missing their Paired Associates Learning Test score.

ANOVA indicates analysis of variance; F, female; M, male; MCI, mild cognitive impairment.

Sex is presented in units.

Echo-planar T2*-weighted volumes were instead registered at rest with the following settings: voxel dimensions 3.28×3.28×6.00 mm; Repetition Time 2 s; Echo Time 50 ms; Field of View 230 mm; flip angle 90 degrees. Two 120-volume runs were obtained, preceded by 20 s of dummy scans, set to allow the scanner to reach electromagnetic equilibrium.

A neuropsychological battery was designed for clinical purposes, with particular focus on those domains which are most sensitive to aging and early-stage neurodegeneration (Fig. 1).

MRI Data Preprocessing

T1-weighted images were processed with the FreeSurfer Image Analysis Suite (http://surfer.nmr.mgh.harvard.edu/) following standard segmentation and parcellation procedures. Morphologic indices were extracted from cortical and subcortical structures. RS-fMRI images were preprocessed using the Statistical Parametric Mapping 8 (Wellcome Trust Centre for Neuroimaging, London, UK) CONN toolbox,[22] in a Matlab R2012a environment (Mathworks Inc., UK). Images were realigned to estimate head-motion vectors, slice-timed to correct for intravolume temporal phasing-out, coregistered with their T1-weighted image, normalized with the echo planar imaging template, smoothed with a 6 mm full-width at half-maximum gaussian filter to minimize noise and residual anatomic discrepancies, partialized of the confounding signal coming from the top 5 orthogonal components estimated from the maps of white matter and cerebrospinal fluid (aCompCor procedure),[23] and band-pass filtered (0.008 to 0.09 Hz).

Feature Definition

A large number of candidate indices were defined from demographic/clinical variables and neurostructural/neurofunctional maps (Fig. 1). Basic demographic information and raw cognitive scores (extracted from clinical neuropsychological tests) were included in this list. Neuroanatomic volumetric indices were extracted from the segmentation and parcellation output. ROI-to-ROI (R2R) indices of functional connectivity were computed from RS-fMRI runs as part of the CONN pipelines. These ROIs were defined based on the anatomically automatic labeled atlas.[24] R2R indices identified aspects of connectivity among pairs of anatomically automatic labeled ROIs. To minimize potential selection bias, and in parallel optimize number of regions, the cerebellum was excluded from the model, as it is characterized by low presence of AD pathology,[25] and is usually considered a reference region in positron emission tomography-based studies. Primary sensorimotor areas were also excluded due to their prolonged preservation in AD.[26] Orbitofrontal and temporopolar regions subjected to signal dropout were excluded too to avoid miscalculations. In total, 2122 indices were extracted: demographics: 3, cognition: 19, sMRI: 84, RS-fMRI: 2016.

Feature Selection

Two machine-learning algorithms were considered. These were the linear and quadratic Fisher discriminant analyses (LDA and QDA, respectively),[27] based on their proneness to being applicable to multiple research contexts, including small-sample scenarios.[28,29] Both classifications were modeled for each set of features. To pursue maximized classificatory accuracy, the classifier with higher accuracy was chosen each time. A feature-selection analysis was then run by testing the performance of the

A

Age	Years of Education	Gender

B

Mini Mental State Examination	*General cognitive screening*
Raven Progressive Matrices	*Visuospatial abstract reasoning*
Digit Cancellation Test	*Visuospatial exploration & speed of processing*
Stroop Test - time interference	*Inhibitory skills*
Stroop Test – error interference	*Inhibitory skills*
Letter Fluency Test	*Phonologically-cued lexical memory access*
Category Fluency Test	*Semantically-cued lexical memory access*
Token Test	*Verbal comprehension*
Similarities Test	*Verbal abstract reasoning*
Confrontational Naming Test	*Lexical memory access*
Digit Span Test - forward	*Verbal short-term memory*
Digit Span Test - backwards	*Working memory*
Paired Associates Learning Test	*Verbal learning*
Prose Memory Test – Immediate	*Verbal long-term memory*
Prose Memory Test – Delayed	*Verbal long-term memory*
Corsi Block Tapping Test	*Visuospatial short-term memory*
Visual Supraspan Test	*Visuospatial learning*
Rey-Osterrieth Figure – Copy	*Visuoconstructive skills*
Rey-Osterrieth Figure – Recall	*Visuospatial long-term memory*

C

FRONTAL	*PARIETAL*
Orbital Gyrus	Superior Parietal Lobule
Rectal Gyrus	Angular Gyrus
Frontomarginal Gyrus	Supramarginal Gyrus
Transverse Frontopolar Gyrus	Precuneus
Superior Frontal Gyrus	
Middle Frontal Gyrus	*OCCIPITAL*
Inferior Frontal Gyrus - Pars Opercularis	Superior Occipital Gyrus
Inferior Frontal Gyrus - Pars Triangularis	Middle Occipital Gyrus
Inferior Frontal Gyrus - Pars Orbitalis	Inferior Occipital Gyrus
Subcallosal Gyrus	Cuneus
Paracentral Lobule	Lingual Gyrus
TEMPORAL	*LIMBIC*
Long Insular Gyrus	Anterior Cingulate Cortex
Short Insular Gyri	Middle-Anterior Cingulate Cortex
Superior Temporal Gyrus - Lateral	Middle-Posterior Cingulate Cortex
Superior Temporal Gyrus - Planum Polare	Posterior-Dorsal Cingulate Cortex
Superior Temporal Gyrus - Planum Temporale	Posterior-Ventral Cingulate Cortex
Middle Temporal Gyrus	Parahippocampal Gyrus
Inferior Temporal Gyrus	*SUBCORTICAL*
Temporal Pole	Thalamus
Subcentral Gyrus	Caudate
Fusiform Gyrus	Putamen
	Globus Pallidus
	Hippocampus
	Amygdala

D

FRONTAL	*OCCIPITAL*
Medial Superior Frontal Gyrus	Superior Occipital Gyrus
Superior Frontal Gyrus	Middle Occipital Gyrus
Middle Frontal Gyrus	Inferior Occipital Gyrus
Inferior Frontal Gyrus - Pars Opercularis	Cuneus
Inferior Frontal Gyrus - Pars Triangularis	Lingual Gyrus
Supplementary Motor Cortex	
Paracentral Lobule	*LIMBIC*
TEMPORAL	Anterior Cingulate Cortex
Insula	Middle Cingulate Cortex
Superior Temporal Gyrus	Posterior Cingulate Cortex
Middle Temporal Gyrus	Parahippocampal Gyrus
Inferior Temporal Gyrus	
Fusiform Gyrus	*SUBCORTICAL*
	Thalamus
PARIETAL	Caudate
Superior Parietal Gyrus	Putamen
Inferior Parietal Gyrus	Globus Pallidus
Supramarginal Gyrus	Hippocampus
Angular Gyrus	Amygdala
Precuneus	

FIGURE 1. List of features and regions included in the study. Demographic features were included in the feature-selection process of all classifiers (A). Each cognitive test is listed together with the cognitive domain it relies on (B). Volumetric features did not include the cerebellum or nonassociative areas but did include regions normally subjected to artefacts during blood oxygen level dependent acquisitions (C). The 64 neocortical patches from which the blood oxygen level dependent signal was extracted were processed to calculate the 2016 resulting patterns of statistical association (D). Volumes and hemodynamic signal were extracted separately for each hemisphere. The exclusion of primary motor, primary sensory, and cerebellar areas allowed the feature-selection procedure to focus on the regions of the brain that are affected by Alzheimer pathology during the preclinical and prodromal stage of the disease, and during the phases of mild and moderate dementia. The regions retained by this methodological choice are involved in high-order processes of cognitive and behavioral function.

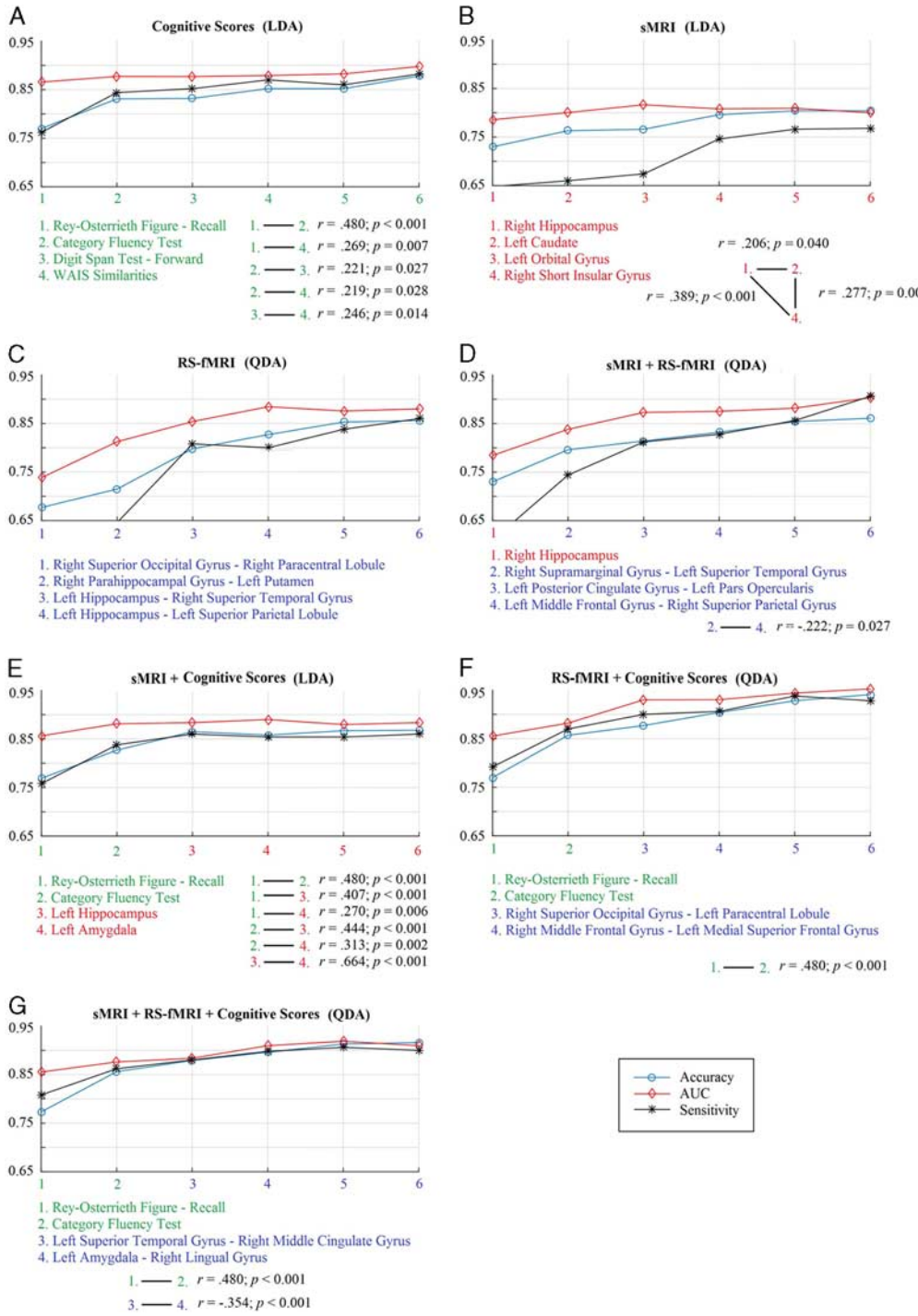

FIGURE 2. The Cognitive (A), sMRI (B), RS-fMRI (C), sMRI+RS-fMRI (D), sMRI+Cognitive (E), RS-fMRI+Cognitive (F), and global sMRI+RS-fMRI+Cognitive (G) classifiers. Accuracy levels are depicted together with measures of sensitivity and area under the curve (AUC). Cognitive, volumetric, and R2R features are indicated in green, red, and blue, respectively (please refer to the online version for color guidance). As the amount of classificatory accuracy decreases with the serial order of the index within the classifier, only the first 4 indices were examined in depth. Correlations among features are indicated below each classifier. AUC indicates area under the receiving-operator curve; LDA, linear Fisher discriminant analyses; QDA, quadratic Fisher discriminant analyze; RS-fMRI, resting-state functional magnetic resonance imaging; R2R, ROI-to-ROI; sMRI, structural magnetic resonance imaging. full color online

chosen classifier as a function of groups of indices. This was achieved via a cost function.[27] The complete data set was subdivided into training and testing subsets using a 10-fold Montecarlo cross-validation. The performance of each classifier was finally evaluated by computing accuracy, area under the receiver-operating-characteristic curve, and sensitivity.

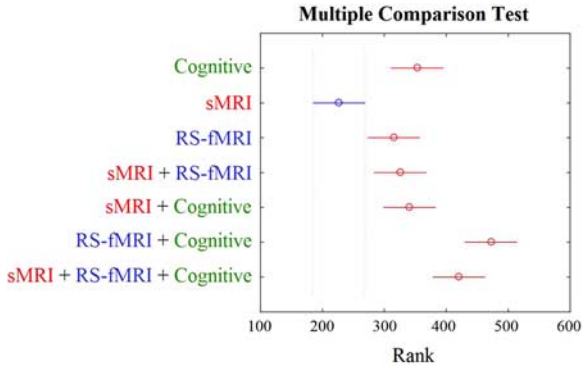

FIGURE 3. Between-classifier Kruskal-Wallis post hoc comparisons. RS-fMRI, resting-state functional magnetic resonance imaging; sMRI indicates structural magnetic resonance imaging (please refer to the online version for color guidance). full color online

Seven classifiers were tested: 3 basic "single-modality" (a, Cognitive; b, sMRI; c, RS-fMRI) and 4 "multiple-modality" classifiers (d, sMRI+RS-fMRI; e, sMRI+Cognitive; f, RS-fMRI +Cognitive; g, sMRI+RS-fMRI+Cognitive). Demographic features were included in all classificatory models. Bonferroni-corrected, post hoc Kruskal-Wallis statistics tested interclassifier differences in accuracy.[29]

RESULTS

The Cognitive classifier (Fig. 2A; LDA) was driven by a test of declarative memory (Rey-Osterrieth Figure—Delayed Recall), and a measure of semantic processing (Category Fluency test). These 2 were responsible for a classificatory accuracy of about 83%. Further tests improved the accuracy rate by an additional 5%. The first volumetric feature selected by the sMRI classifier (Fig. 2B; LDA) was the right hippocampus, followed by the left caudate and the left orbital gyrus. These 3 features approached a 77% accuracy, reaching 80% with additional indices. The RS-fMRI classifier (Fig. 2C; QDA) overstepped an 85% accuracy plateau after 5 indices. These were patterns of R2R connectivity widespread across various regions of the brain, but heavily hinging upon mediotemporal regions (3 of 5 indices). The mixed sMRI+RS-fMRI classifier (Fig. 2D; QDA) obtained performance levels equal to 85% accuracy after 5 indices. The volume of the right hippocampus was selected as the most accurate, followed by R2R connectivity of various associative (prefrontal, parietal, and temporal) cortices. As with the cognitive classifier, the remaining 3 mixed classifiers were reliant on declarative memory and semantic processing as the 2 leading features. In the sMRI+Cognitive classifier (Fig. 2E; LDA) these 2 indices reached an 83% accuracy, marginally improved by volumetric properties of the left mediotemporal complex. In the RS-fMRI+Cognitive classifier (Fig. 2F, QDA), and in the global sMRI+RS-fMRI+Cognitive classifier (Fig. 2G, QDA) the accuracy of the 2 tests reached an accuracy of over 85%, further improved by additional R2R indices. In the global classifier the accuracy was raised to 90% with the addition of indices characterizing left temporal connectivity. Conversely, in the RS-fMRI +Cognitive classifier the accuracy was further enhanced up to 94% with the contribution of 2 indices of widespread connectivity.

The comparison between classifiers revealed that the RS-fMRI+Cognitive classifier was, by far, the most accurate ensemble, accounting for a significantly more accurate classification than 5 of the other classifiers. Vice versa, the sMRI classifier was the least accurate, performing significantly worse than any other classifier (Fig. 3).

For each classifier, the performance of the less accurate classification methods (LDA or QDA) was associated with 2% to 3% less accuracy than the rates of those described above. Nonetheless, these were reliant on comparable sets of features (the recall of Rey-Osterrieth Complex Figure and Category Fluency, the volume of the right hippocampus, and the connectivity of mediotemporal regions).

Selected post hoc analyses were run to understand the clinical importance of these features driven by and in support of possible interpretational frameworks (Fig. 4). Within the cognitive classifier a significant difference existed

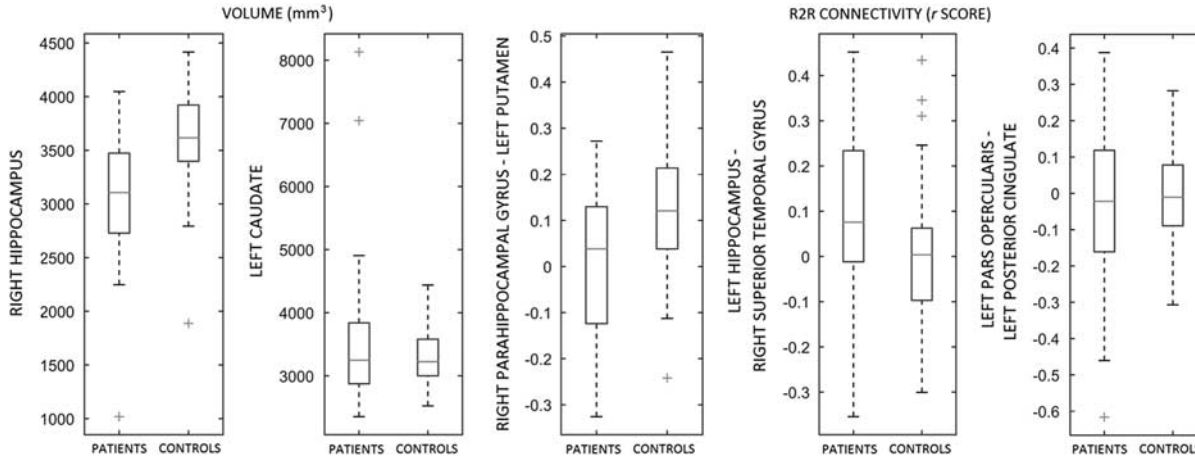

FIGURE 4. Selected post hoc analyses. Between-group comparisons (*t* test statistics) were run to explore the group-level differences of the main features included in the classifiers. MCI patients had significantly larger volumes in the right hippocampus but no difference in the left caudate nucleus. Moreover, patterns of connectivity showed a trend in either direction and, as exemplified by the association between the left posterior cingulate and the left pars opercularis, did only differ in the pattern of dispersion. MCI indicates mild cognitive impairment; R2R, ROI-to-ROI.

between the 2 diagnostic groups on the delayed recall scores on the Rey-Osterrieth Figure and on the Category Fluency Test (both $P < 0.001$). No significant difference was present; however, between the 2 groups on the 2 subsequent tests (Digit Span—forward and the similarities subtest of the Wechsler Adult Intelligence Scale). Moreover, a significant correlation was found between the delayed recall scores on the Rey-Osterrieth Figure and the delayed recall scores on the Prose Memory test (partial correlation correcting for age and education levels, $P = 0.000006$), and between the delayed recall scores on the Rey-Osterrieth Figure and the volume of the right hippocampus ($P = 0.00013$).

Within the structural classifier, a significant difference was found between groups solely for the volume of the right hippocampus, and the volumes of the 2 hippocampi and caudate nuclei were highly correlated ($P = 4.90e\text{-}30$ and 4.20e-52, respectively).

As most of the RS-fMRI R2R indices featured the connectivity of mediotemporal areas, their between-group directionality was explored. The association tended to be larger in controls for some of the features (eg, the second feature: right parahippocampal gyrus—left putamen R2R connectivity), and larger in patients for others (eg, the third feature: left hippocampus—right superior temporal gyrus R2R connectivity).

In the mixed sMRI+RS-fMRI classifier the first 2 R2R features were explored further. A 0.2 z difference from controls was seen for the first R2R (temporal-parietal) feature in patients. In contrast, the correlation between the posterior cingulate cortex and the left pars opercularis was close to 0 in both groups, but showed a larger dispersion in the patient group.

Finally, Pearson correlations were run to explore the association among the top features, within each classifier. Variable results were found, with cognitive and sMRI features showing significant correlations, and RS-fMRI indices tending instead to be statistically independent from each other (Fig. 2).

DISCUSSION

AD triggers a large number of alterations to brain structure, brain connectivity, and cognitive function. Partly, this is the result of a global process of decline which, homogenously, affects a large number of regions, circuital pathways, and cognitive domains (ie, global atrophy and ventricular enlargement, global loss of network connectivity and regional isolation, global cognitive decline). What looks like a general trend, however, can be broken down into separate processes. In AD, studies have highlighted that disease progression involves a number of separate routes. For instance, loss of posteromedial metabolism and atrophy in the mediotemporal complex seem to be driven by distinct mechanisms.[30] Similarly, changes in patterns of connectivity in crucial network pathways are governed by disease-specific, compensatory, and maladaptive mechanisms, which can induce decreases but also increases in the resulting phenotype.[31,32] The extrapolation of independent disease mechanisms can be helpful in clinical settings. For example, there are studies which highlight the importance of exploring mechanisms of both declarative and semantic memory for an early diagnosis of AD, as semantic processing is severely down-regulated in AD, but not significantly disrupted by the normal processes of aging.[33] On this note, the use of machine-learning algorithms for classification purposes is an excellent approach to clarify the diagnostic importance of features extracted from structural and functional neuroimaging. As commented below, however, the particularity of this approach lies in the elimination of any redundancy expressed by features significantly correlated with one another. The resulting combination of variables, therefore, captures distinct aspects of classification, and, thus, of disease.

RS-fMRI Improves Classification

A look at the quantitative aspects of classificatory performance reveals that the sMRI classifier was the least accurate. This indicates that morphometric biomarkers are not as effective as fMRI or cognitive features at detecting abnormalities in the presence of MCI. We argue that, as hippocampal and brain volumes are in fact also influenced by nonpathologic aging,[34] they are unsuitable to provide classificatory specificity.

Classifiers based on cognitive features performed very well. This is necessarily due to the fact that the standard of truth (ie, "patient" or "control") was heavily based on the presence of cognitive impairment measured with cognitive tests.

The most accurate classifications were obtained when RS-fMRI features were included in the feature-selection process. The performance of the RS-fMRI classifier did in fact not differ from that of the Cognitive classifier. In addition, RS-fMRI features improved classification of both sMRI and cognitive features. One possible reason behind such good performance may be the large number (2016) of available RS-fMRI features. This should be seen as an advantage enabled by RS-fMRI modalities (rather than a methodological imbalance), as RS-fMRI offers the opportunity of exploring properties of the blood oxygen level dependent signal which are not absolute (ie, related only to a specific voxel or ROI), but relative (ie, reflective of the relationship between 2 voxels or ROIs). These dynamic characteristics are profoundly associated with the basic processes of brain functioning, as task performance is supported by the interactive coactivation/codeactivation of multiple structures.

Each Classifier as Informant of Distinct Mechanisms

A closer, qualitative look at each classifier allows the clarification of: (1) how useful machine-learning algorithms are to extract classificatory information, and (2) how this method helps the understanding of the various types of mechanisms which may separate patients from controls.

As for the Cognitive classifier, the first feature was a measure of declarative memory (the delayed recall of the Rey-Osterrieth Figure), a domain well known to be severely affected in AD. Although cognitive assessment featured a second measure of long-term declarative memory (the delayed recall of the Prose Memory test), this variable was not chosen as part of the classifier. We argue that the significant correlation found between the 2 memory tests translates into comparable classificatory accuracies, hence the non-necessity of including both. In contrast, the performance on a measure of semantic processing (the Category Fluency test) accounted for an exclusive and relevant amount of variability. Declining semantic processing is one of the major features of various forms of neurodegeneration, and occurs as a result of compromised circuits sustained by regions that are anatomically distinct from those in support of declarative memory.[35] By relying on the same argument, we speculate that the global classifier did not include both the performance on the delayed recall of the Rey-Osterrieth Figure and the volume of the right hippocampus (the "top"

cognitive and MRI-based features, respectively) because of a conceptual association between the 2 variables.[36]

The sMRI classifier was heavily reliant on the right hippocampus in our sample, whereas the left hippocampus, presumably because of a very high interhemispheric correlation coefficient, was not included. The second volumetric feature was the left caudate nucleus (presumably contributed by both caudate nuclei, given the large intrahemispheric correlation). Although the volume of the right hippocampus was significantly smaller in the group of patient, no significant between-group difference emerged for the left caudate. It is interesting to note how features with no between-group differences may yield classificatory relevance. We argue that there might be structures subjected to minor morphometric changes, which, however, are more distinctively related to cognitive impairment than any more extensive morphometric dysregulation located elsewhere. On this note, studies on human and primate brains show that neuronal and synaptic densities are not homogenous across the entire cortex.[37,38] Small group differences in a region with high cell density or sustaining a crucial function might have profound biological implications. Dopaminergic neurons represent an example of this mechanism in Parkinson disease, as they are a minimal portion of the total number of nervous cells, but they serve paramount purposes. In this respect evidence does shows that the caudate manifests volumetric shrinkage in AD,[39] whereas this does not occur in healthy aging.[34] These findings show that the caudate alterations seen in patients, albeit not reaching statistical significance in any specific direction, seem to be independent from mediotemporal modifications, yet conceptually relevant for the diagnosis of MCI.

The RS-fMRI classifier was profoundly based on the connectivity of the left and right hippocampal formation. The first feature represented the R2R pathway accounting for the single largest portion of variability in our sample. The subsequent 4 features all entailed independent aspects of mediotemporal connectivity. Since the earliest histopathologic descriptions, AD has been described as a disease that causes a computational isolation of the hippocampus.[40] Loss of hippocampal and parahippocampal connectivity would be the in vivo equivalent of this process. In addition, one of the R2R features showed a trend toward the opposite direction, with patients having increased hippocampal-temporal connectivity. In line with the evidence of increased hippocampal metabolism shown during the MCI stage,[32] we hypothesize that up-regulated connectivity in patients may be the result of neuroplastic modifications triggered by the early stages of hippocampal disconnection, and that the RS-fMRI classifier is suitable to capture disease mechanisms as well as neuroplastic responses. These latter would in all likelihood not be recordable by morphometric acquisitions, which reflect instead gross anatomy, well known to be more resistant to neuroplastic alterations.

We then included a mixed sMRI+RS-fMRI classifier to understand whether the sole information extracted from an MRI protocol could be exploited clinically. Hippocampal volumes were confirmed as the most informative feature. Decreased connectivity (a 0.2 average drop in the strength of the correlation coefficient) between temporal and parietal region improved this classification. Interestingly, for the third feature (posterior cingulate to Broca area), the *r* coefficient was close to 0 in both groups (indicating no association). In this case, the 2 groups differed in the dispersion levels, suggesting that the informative aspect for this pathway might be the presence of an association (regardless of the directionality) in a pathway where an association would normally not exist.

The mixed sMRI+Cognitive classifier was constructed based on the combination of features that are usually at disposal of the clinician (a cognitive assessment and an anatomic brain scan). The results are perfectly in line with the typical pattern of clinical features that drives a diagnosis of early-stage neurodegeneration, as the selected features are measures of declarative memory and mediotemporal volumes.

The RS-fMRI+Cognitive classifier was the top-performing one. When the analysis of declarative memory is flanked by measures of connectivity, the classification approaches optimal levels (accuracy ~94%) and outperforms the support provided instead by sMRI. The superior performance of this classifier might reflect the qualitatively different disruption caused by AD neurodegeneration on brain function, leading often to compensatory change in controls and maladaptative alteration in the early stage of neurodegeneration.[31]

Finally, the outcome of the global classifier confirmed that the characterization of cognitive profiles (presence of declarative memory and semantic processing deficits) was by far the most accurate predictive formula for classifying patients. R2R features contributed to improving the accuracy by highlighting the role played by various aspects of the limbic system, and temporooccipital areas.

Limitations

Despite the protection toward bias offered by a data-driven approach and a sample of comparable or larger size than that of other studies,[16–20] the outcome is still the result of feature and algorithm definition. Although we selected "standard" cognitive tests and segmentation/parcellation atlases, and 2 basic machine-learning algorithms, we cannot rule out the possibility that other methodological choices might have yielded slightly different patterns of findings. This, however, would not undermine the core findings and interpretations. Moreover, the sets of cognitive, neuroanatomic, and neurofunctional variables are qualitatively different from one another, for example, in their number, in the presence of a numerical ceiling, or in their directionality (as patients may show either decreased or increased RS-fMRI connectivity, but only an impoverishment of cognition and brain structure, see Table 2 for the most distinctive anatomic and R2R). Inevitably, feature selection will be affected by these different properties. As a consequence, comparisons of classifiers will be meaningful as far as quantitative performance is concerned, but any analysis focusing on confronting different types of features has to be interpreted with caution. Post hoc inter-feature correlations are in line with the presence of such qualitative differences, as, for instance, most cognitive features (fewer in number) were mutually correlated, determining a certain degree of collinearity, whereas RS-fMRI indices (many in number) were unrelated with one another.

Clinical Usefulness of Machine-learning Methods

In conclusion, these findings indicate that RS-fMRI R2R connectivity improves diagnostic classification of patients with MCI, and outperforms the accuracy of sMRI, which was profoundly reliant on the importance of hippocampal volumes. A careful look at each classifier revealed that machine-learning approaches, by circumventing

TABLE 2. Distinctive Neuroanatomic and Neurofunctional Characteristics (expressed as means and standard deviations in parentheses) of the 2 Diagnostic Groups

Neuroimaging Variables	Healthy	MCI	P $F_{Corrected}$
sMRI Index—significant difference: controls > patients			
Left hippocampus	3508.65 (397.22)	3038.34 (490.47)	<0.001
Right hippocampus	3598.68 (425.13)	3092.91 (554.34)	<0.001
Left parahippocampal gyrus	3288.38 (476.96)	2908.46 (589.22)	0.004
Right lingual gyrus	4041.22 (563.28)	3646.56 (555.56)	0.002
RS-fMRI Index—significant difference: controls > patients			
Right middle frontal gyrus—left insula	0.0450 (0.17)	−0.0599 (0.16)	0.002
Right inferior frontal gyrus, pars triangularis—right thalamus	0.1534 (0.18)	0.0413 (0.16)	0.003
Left supplementary motor cortex—left globus pallidus	0.2330 (0.12)	0.1221 (0.14)	<0.001
Left posterior cingulate cortex—left inferior temporal gyrus	0.2131 (0.16)	0.0928 (0.18)	0.002
Left parahippocampal gyrus—right paracentral lobule	0.0681 (0.14)	−0.0522 (0.17)	0.001
Right parahippocampal gyrus—left putamen	0.1159 (0.14)	0.0148 (0.15)	0.004
Left cuneus—left paracentral lobule	0.1043 (0.19)	−0.0123 (0.22)	0.005
Left cuneus—right putamen	0.0226 (0.17)	−0.0722 (0.17)	0.004
Right cuneus—right paracentral lobule	0.1628 (0.18)	0.0217 (0.22)	0.003
Right cuneus—right putamen	0.0741 (0.16)	−0.0495 (0.20)	<0.001
Left occipital superior gyrus—right putamen	0.0254 (0.17)	−0.0874 (0.17)	0.002
Right fusiform gyrus—left supramarginal gyrus	0.0425 (0.17)	−0.0452 (0.17)	0.001
Right fusiform gyrus—left superior temporal gyrus	0.0815 (0.19)	−0.0370 (0.18)	0.004
Right fusiform gyrus—right superior temporal gyrus	0.1791 (0.22)	0.0423 (0.18)	<0.001
Right fusiform gyrus—right inferior frontal gyrus, pars opercularis	−0.0269 (0.16)	−0.1087 (0.17)	0.003
Right fusiform gyrus—right inferior frontal gyrus, pars triangularis	−0.0028 (0.15)	−0.0920 (0.20)	0.005
Left precuneus—left middle temporal gyrus	0.3054 (0.17)	0.1787 (0.20)	0.001
Left precuneus—left inferior temporal Gyrus	0.1434 (0.18)	0.0508 (0.20)	0.002
Right caudate—left middle temporal Gyrus	0.0244 (0.15)	−0.0770 (0.16)	0.004
Left putamen—left thalamus	0.2350 (0.16)	0.1365 (0.17)	0.004
Left putamen—right middle temporal Gyrus	0.0859 (0.15)	0.0001 (0.13)	0.003
RS-fMRI Index—significant difference: patients > controls			
Left middle frontal gyrus—right superior medial frontal gyrus	0.1316 (0.23)	0.2526 (0.21)	0.005
Right middle frontal gyrus—left superior medial frontal gyrus	−0.0267 (0.25)	0.1617 (0.24)	<0.001
Right middle frontal gyrus—right superior medial frontal gyrus	0.2337 (0.27)	0.4066 (0.22)	0.001
Left inferior temporal gyrus—right middle frontal gyrus	0.0029 (0.19)	0.1301 (0.21)	0.001
Left inferior temporal gyrus—right inferior frontal gyrus, pars triangularis	0.0064 (0.19)	0.1210 (0.21)	0.004
Left hippocampus—left superior temporal gyrus	0.0362 (0.17)	0.1791 (0.16)	<0.001

Between-group differences in sMRI and RS-fMRI indices were analyzed with ANOVAs, correcting for age. As a Bonferroni correction was judged too conservative for such a large number of statistical comparisons (n = 2100), a still relatively strict $P = 0.005$ was used. Of the entire set of indices, only 4 sMRI and 27 RS-fMRI indices survived this threshold and were reported in the table.

ANOVA indicates analysis of variance; MCI, mild cognitive impairment; RS-fMRI, resting-state functional magnetic resonance imaging; sMRI, structural magnetic resonance imaging.

feature-to-feature statistical redundancy, generate classifiers in which each feature accounts for an independent portion of classificatory accuracy, presumably in reflection of separate disease mechanisms. These might manifest as decrease/increase in R2R correlation (and these differences are often very small and not significant), or in the presence of a correlation between 2 otherwise uncorrelated areas. In addition, between-group volumetric differences do not seem to scale to a common denominator, as minimal differences in specific structures might be more relevant than larger differences elsewhere. These alterations might represent an important source of clinical information and have to be further explored to be implemented in neurological settings. The nature of these findings suggest that clinically relevant alterations seen in brain function of MCI patients might be quite subtle and not potentially inferable from group-based analyses.

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Volume and Connectivity of the Ventral Tegmental Area are Linked to Neurocognitive Signatures of Alzheimer's Disease in Humans

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Abstract.
Background: There is an urgent need to identify the earliest biological changes within the neuropathological cascade of Alzheimer's disease (AD) processes. Recent findings in a murine model of AD showed significant preclinical loss of dopaminergic neurons in the ventral tegmental area (VTA), accompanied by reduced hippocampal innervation and declining memory. It is unknown if these observations can be translated in humans.
Objective: We tested the hypothesis that VTA volume is associated with the typical clinical markers of AD in a cohort of patients and healthy controls.
Methods: Structural and resting state functional MRI scans, and neuropsychological scores were acquired for 51 healthy adults, 30 patients with a diagnosis of mild cognitive impairment, and 29 patients with a diagnosis of AD dementia. VTA volume was quantified together with other control nuclei. The association between nuclei volume, hippocampal size, memory performance, and linguistic-executive skills was tested. The effect of VTA functional connectivity was also tested.
Results: VTA size, but not of control nuclei, yielded a strong association with both hippocampal size and memory competence (but not linguistic-executive performance), and this was particularly strong in healthy adults. In addition, functional connectivity between the VTA and hippocampus was significantly associated with both markers of AD.
Conclusion: Diminished dopaminergic VTA activity may be crucial for the earliest pathological features of AD and might suggest new strategies for early treatment. Memory encoding processes may represent cognitive operations susceptible to VTA neurodegeneration.

Keywords: Alzheimer's disease, cognitive dysfunction, dopaminergic neurons, early diagnosis, functional neuroimaging, gray matter, hippocampus, memory, mild cognitive impairment, neuroimaging, tegmentum mesencephali, ventral tegmental nucleus

INTRODUCTION

The epidemiological and economic burden of Alzheimer's disease (AD) increases [1], but the exact mechanisms by which the initial neuropathological changes are triggered are still elusive. The "classic" amyloid-β cascade hypothesis posits that it is the abnormal accumulation of this protein in parenchymal regions that induces all subsequent changes in neural structure and function seen in AD. This hypothesis is currently at the center of a scientific debate, with evidence in its support [2], and research conclusions, which, instead, do not sustain its claims [3]. A crucial aspect in the identification of the ontogenesis of the disease is certainly a progressive shift towards the characterization of the earliest preclinical

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stages of AD (i.e., when individuals are in their early adulthood, or even earlier). This has been pursued both to clarify the causing mechanisms, but also to find an early disease marker, that may be of assistance in the diagnostic process. Although focusing on the genetic form of AD is by far the most convenient approach to study preclinical AD in humans (young carriers of a mutation in one of the AD-related genetic loci will inevitably go on developing the disease), the study of sporadic preclinical AD (not necessarily bound to its genetic forms) is instead a much more effortful enterprise that demands large cohorts and long study durations. On this note, only a few studies have identified variables that could be exploitable in a clinical setting and, at the same time, shed light on the mechanism behind the neurotoxic cascade of AD. A prominent finding emerged from this type of research is that showing that an impoverishment of lexical-semantic abilities during early adulthood is a significant predictor of AD pathology at postmortem [4]. A second finding has emerged from detailed histological analysis of brain tissue: non fibrillar precursors of abnormal TAU protein are detected in early adulthood (i.e., "pre-tangle" material) in brainstem nuclei, especially in the locus coeruleus [5]. In an attempt to identify a preclinical marker, we tested a hypothesis derived from the results of a study published very recently. In their manuscript, Nobili and colleagues found that, in a mouse model of the disease, very early anatomical changes are present in a subcortical brain region rich in dopaminergic neurons, the ventral tegmental area (VTA), or ventral tegmentum [6]. Specifically, neuronal loss seems to be present in this area prior to any deposition of amyloid-β plaques. Moreover, this is accompanied by reduced dopaminergic innervation to the hippocampus, and decreased memory performance [6]. Although this was found in a group of transgenic mice carriers of an AD-related genetic mutation, the principle that a dopaminergic process may be a prime mechanism that contributes to triggering the neurotoxic cascade would be a valid principle in any form of AD. On these grounds, we thus transposed and tested this hypothesis in a sample of humans.

We hypothesized that the size of the VTA, estimated with a volumetric index, obtained from magnetic resonance imaging, would be significantly associated with the size of the hippocampus and with performance on a test of episodic memory. We also tested whether VTA functional connectivity would co-vary with memory performance and hippocampal volume.

MATERIAL AND METHODS

Participants

A cohort of 110 individuals was included in this study. These had been recruited at the Royal Hallamshire Hospital (Sheffield, UK), as part of the EU-funded research initiative Virtual Physiological Human: DementiA Research Enabled by IT (http://www.vph-dare.eu/; see Acknowledgments section). Of those included in this cohort, fifty-one were heathy adults free from neurological symptoms or cognitive complaints. Other twenty-nine were patients with a clinical diagnosis of mild/moderate AD dementia. The remaining thirty participants were patients with a diagnosis of mild cognitive impairment (MCI) of the single-domain amnestic type ($n = 1$), multiple-domain amnestic type ($n = 12$), single-domain non-amnestic type ($n = 7$), multiple-domain non-amnestic type ($n = 10$), that could not be accounted for by neurovascular, psychiatric, metabolic or traumatic reasons [7]. The clinical profile of these patients (detailed by a senior neurologist and a senior clinical neuropsychologist) was strongly indicative of underlying AD pathology as the main etiology causing their symptoms, and the diagnostic criteria for MCI due to AD were applied to classify each of these 30 patients as prodromal AD [8]. Specifically, all patients had been followed up clinically at regular intervals for at least two and a half years for the confirmation of the diagnosis.

Each participant completed a magnetic resonance imaging (MRI) protocol (see subsequent section) and an extensive battery of cognitive tests, to comply with study criteria and clinical profiling (illustrated in Table 1). Of these, two indices of cognitive competence were extracted from the battery of tests: the performance on the Prose Memory test (the average of the immediate and delayed recall scores, as well as immediate and delayed recall scores taken separately) as a measure of verbal episodic memory [9], and the performance on the Letter Fluency test as a measure of language and executive functioning not reliant on the hippocampus [10]. Raw scores on these two tests were converted into z scores based on the mean and standard deviation of the entire cohort, with the following formula $z_x = (x_i - \mu)/\delta$. Mini-Mental State Examination (MMSE) scores [11] were also extracted from each assessment.

This study received ethical approval from the Yorkshire and Humber Regional Ethics Committee,

Table 1
Variables included in this study as distributed across the different diagnostic cohorts

Variable	Healthy ($n=51$)	MCI ($n=30$)	AD Dementia ($n=29$)	Group Differences (p)*	Bonferroni-Corrected *Post Hoc* Significance
Demographic Characteristics					
Age (y)	61.96 (16.38)	64.67 (10.12)	63.97 (9.52)	0.638	N/A
Education (y)	14.88 (3.18)	12.90 (2.99)	12.00 (2.34)	<0.001	Healthy > MCI/AD
Gender Ratio (f/m)	34/17	15/15	9/20	0.008	Healthy ≠ AD
Mini-Mental State Examination	28.24 (1.79)	25.63 (2.16)	19.24 (3.18)	<0.001	Healthy > MCI > AD
Cognitive Indices					
Prose Memory – Average Recall (z score)	0.84 (0.42)	–0.28 (0.67)	–1.19 (0.51)	<0.001	Healthy > MCI > AD
Prose Memory – Immediate Recall (z-score)	0.82 (0.58)	–0.29 (0.60)	–1.14 (0.50)	<0.001	Healthy > MCI > AD
Prose Memory – Delayed Recall (z-score)	0.82 (0.34)	0.26 (0.77)	–1.18 (0.57)	<0.001	Healthy > MCI > AD
Letter Fluency (z-score)	0.66 (0.79)	–0.39 (0.77)	–0.75 (0.79)	<0.001	Healthy > MCI/AD
Neuroanatomical Indices					
VTA Ratio	5.26e-03 (7.19e-04)	5.23e-03 (6.65e-04)	4.92e-03 (7.36e-05)	0.107	N/A
RN Ratio	3.38e-03 (4.72e-04)	3.47e-03 (3.91e-04)	3.30e-03 (4.43e-04)	0.317	N/A
SN Ratio	1.55e-03 (2.13e-04)	1.51e-03 (2.09e-04)	1.45e-03 (2.13e-04)	0.129	N/A
Gray Matter Ratio	0.44 (0.06)	0.43 (0.05)	0.37 (0.05)	<0.001	Healthy/MCI > AD
Hippocampal Ratio (STEPS)	1.77e-03 (2.25e-04)	1.70e-03 (2.66e-04)	1.46e-03 (3.49e-04)	<0.001	Healthy/MCI > AD

*One-way *ANOVA* and *chi-square* tests were used. MCI = mild cognitive impairment. Aside from Gender Ratio, means and standard deviations are indicated for each variable. Ratio indices of each subcortical nucleus was calculated based on the volume of brainstem space. Hippocampal ratios reported here are based on the use of the STEPS procedure.

Ref No: 12/YH/0474. Written informed consent was obtained from all participants prior to enrollment.

MRI acquisition

Each participant underwent an MRI research protocol (Philips Achieva, 3 T) inclusive of anatomical and functional image sequences. Of these, T1-weighted and resting-state fMRI images were the acquisition types suitable to address the planned experimental question.

T1-weighted images were acquired with the following parameters: voxel size: 0.94 mm × 0.94 mm × 1.00 mm; TR 8.2 s; TE 3.8 s; field of view: 256 mm; matrix size: 256 × 256 × 170.

Resting-state fMRI images were based on 125 volumes, acquired with the following specifications: TR 2.6 s, TE 35 ms, flip angle 90°, voxel dimensions 1.80 × 1.80 × 4.00 mm, field of view 230 mm, 35 slices per volume.

MRI processing

Image processing was carried out with Matlab (Mathworks Inc., UK) and Statistic Parametric Mapping 12 (Wellcome Trust Centre for Neuroimaging, London, UK). The T1-weighted MRI sequence was processed with standard voxel-based morphometry [12]. Images were initially segmented to separate the maps of gray matter, white matter, and cerebrospinal fluid, were registered to the Montreal Neurological Institute anatomical template, and were smoothed with an 8 mm full-width at half maximum Gaussian kernel. The volumes of the three tissue class maps in the native space were quantified using the "get_totals" Matlab function (http://www0.cs.ucl.ac.uk/staff/g.ridgway/vbm/get_totals.m). These were added up to obtain the total intracranial volume and, in turn, the global gray matter ratio.

Volumes of interest were drawn using the PickAtlas toolbox and the Brodmann's atlas [13]. The VTA was defined in the Montreal Neurological Institute space as a spherical volume of 3 mm radius centered at $x=0$, $y=-16$, $z=-7$, as implemented in previous research [14, 15]. Additional regions were selected as methodological control (Fig. 1). These were the red nucleus (RN), based on its proximity to the VTA, and the substantia nigra (SN), another region rich in dopaminergic neurons which had been found to not play any role in the pre-plaque stage in the study by Nobili and colleagues [6]. Mean gray matter signal intensity was then extracted from each volume of interest with MarsBaR [16], as done in previous research (e.g., [17]). Since regional volumes are influenced by head size, these were normalized to ratios of the brainstem. To do so, a brainstem mask was

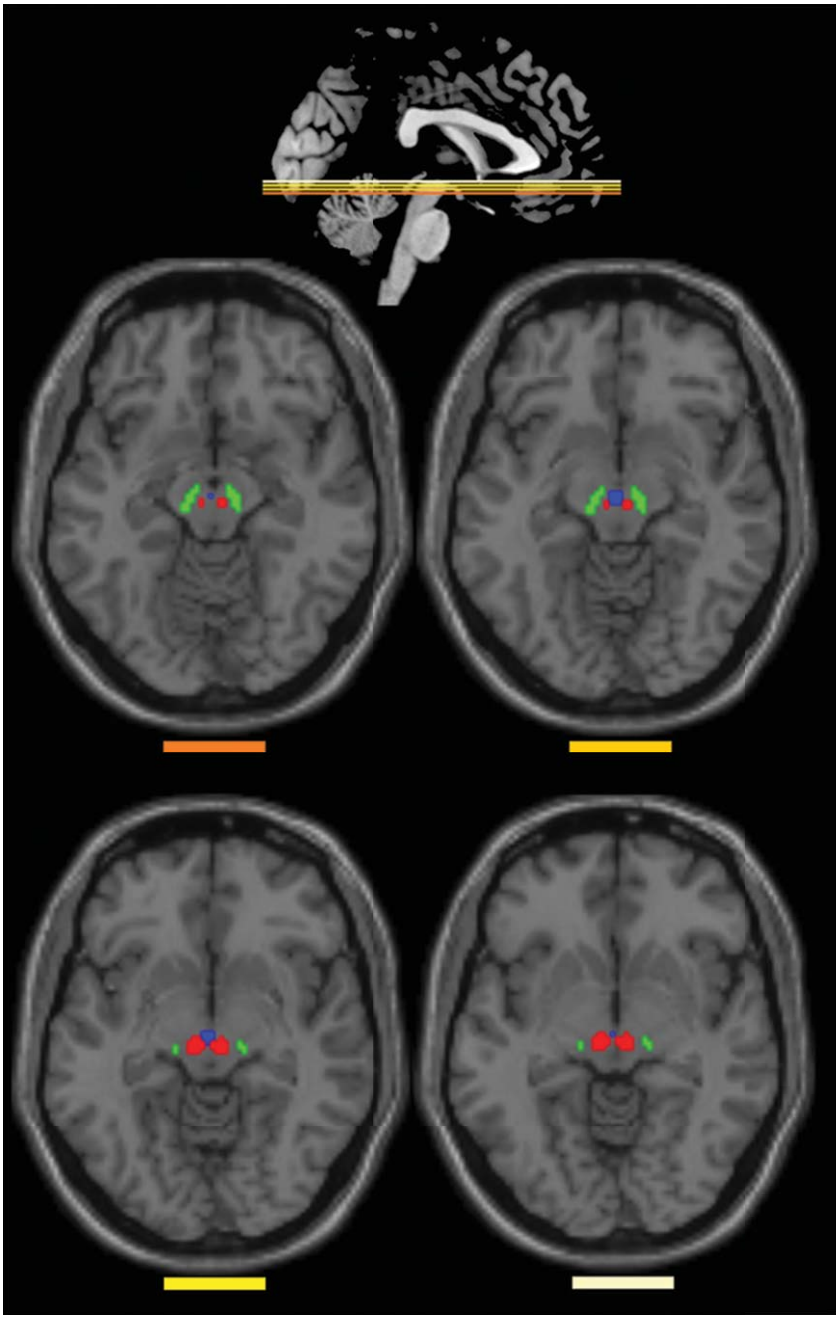

Fig. 1. The regions included in this study identified by masks superimposed to the MNI anatomical template. The RN, SN, and VTA are shown in red, green, and blue, respectively. Each axial slice (Montreal Neurological Institute coordinates from the top left: $z = -12, -10, -8, -6$, clockwise) is identified on the orthogonal view by the shade of yellow/orange. For color coded regions, please refer to the online version.

created using PickAtlas, and volumes were extracted using the "get_totals" script.

Hippocampal volumes were calculated using Similarity and Truth Estimation for Propagated Segmentations (STEPS), an automated procedure that segments the hippocampus from native-space anatomical images based on multiple templates (http://cmictig.cs.ucl.ac.uk/nifty). STEPS outperforms other methodologies on the segmentation of the hippocampus, and generates results that closely resemble those of manual segmentation [18]. Figure 2 illustrates two examples of the use of this proce-

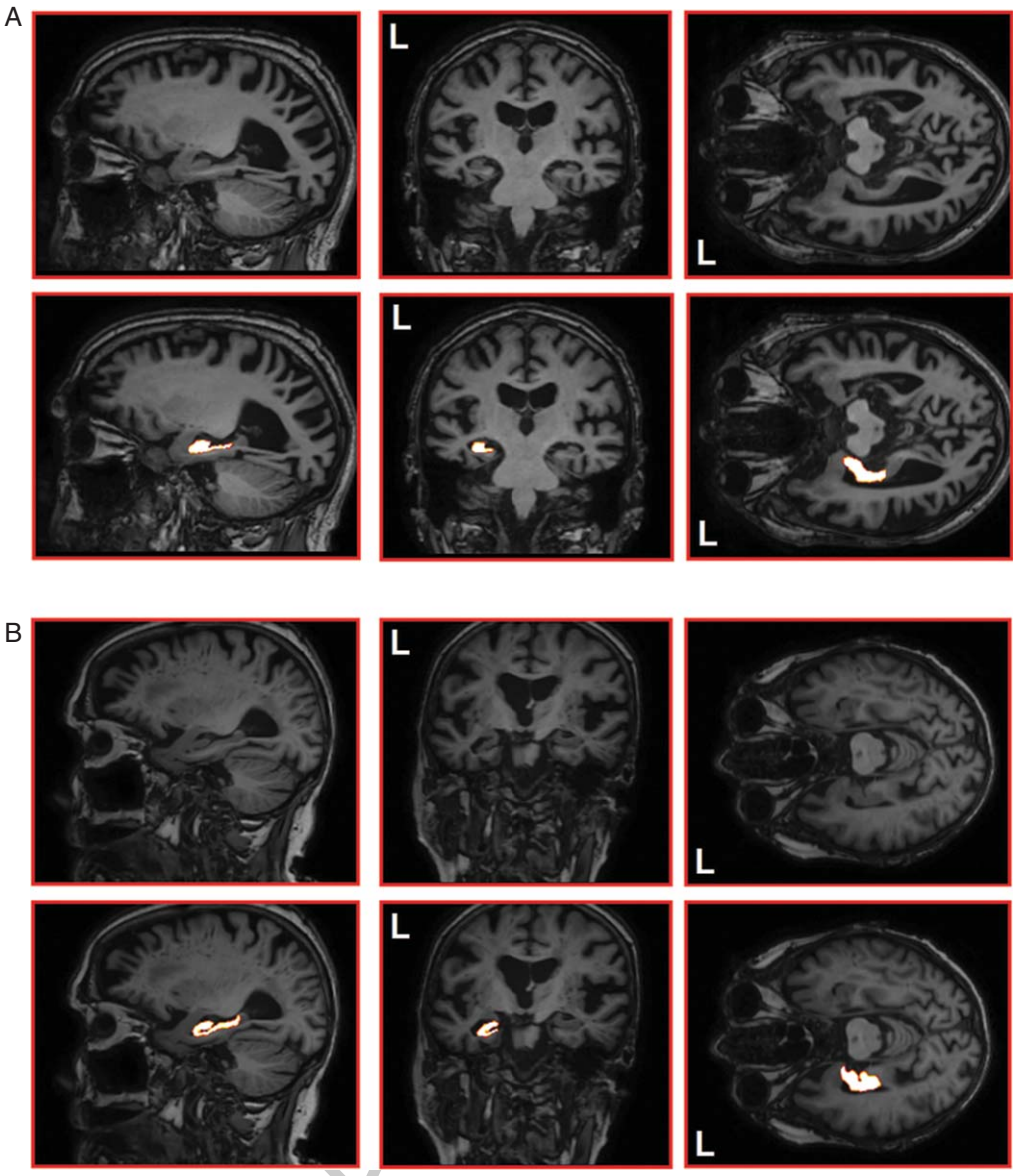

Fig. 2. Two examples of hippocampal segmentation using STEPS. Hippocampal volumes were calculated based on the T1-weighted image in its native space. These two examples show the segmentation of the left hippocampus of a patient with AD dementia (A) and a healthy control (B). Slices are shown with and without the hippocampal overlay.

dure. Each of the 110 outputs was visually inspected for quality control. The "get_totals" Matlab function was used to convert the output files into a volumetric index. This was partialized based on total intracranial volumes, and left and right hippocampal ratios were averaged to obtain a global hippocampal ratio.

Resting state T2* images, indexing the haemodynamic properties of the brain, were preprocessed following a standardized methodology, as described elsewhere (e.g., [19]). Briefly, raw images were initially slice-timed and realigned to even out temporal and spatial variability in the acquisition process. A normalization followed, during which scans were registered to the Montreal Neurological Institute space. A band-pass filter was then applied to retain the range of frequencies relevant for neural signal (0.01–0.1 Hz). Finally, filtered scans were smoothed with a 6 mm full-width at half-maximum Gaussian kernel. The hemodynamic timecourse within each voxel was modelled as a function of the signal of the VTA, regressing out the signal coming from white

matter and cerebrospinal fluid, and in-scanner motion parameters.

Modelling

Non-parametric correlation models were run to test the association between each anatomical ratio (VTA ratio, RN ratio, and SN ratio) and the neurocognitive features of AD (hippocampal ratio, memory performance, and, as control measure, linguistic-executive performance). The threshold for statistical significance of the *Spearman's rho* coefficients accounted for nine (3 nuclei × 3 models) independent correlations ($p < 0.005$). Since healthy controls were significantly more educated than patients, education-corrected *Spearman's rho* coefficients were also calculated. MMSE scores were added as second covariate in the models testing the correlation between hippocampal ratio and the size ratio of the nuclei. Since a very strong correlation existed between MMSE and Prose Memory scores ($rho = 0.788$, $p = 1.64e{-}24$), the correlation models testing the association between memory performance and VTA ratio were not corrected for MMSE.

To reach a better understanding of the structural and functional relation between each nucleus and the rest of the brain, other analyses were run. First, the structural covariance of the VTA and the other nuclei was explored. This served to understand what pattern of regions tend to covariate in volumetric terms with each nucleus. Voxel-by-voxel regression models were carried out across the entire cohort, in which gray matter maps were modelled as a function of the size of each nucleus. The score on the MMSE was used as a correction factor for these analyses.

Second, maps of VTA functional connectivity were analyzed. This was done as a function of the normalized hippocampal ratio and memory performance, in the entire cohort and within each diagnostic group. Age, education levels, and gray matter ratios were included as covariates in each model. Scores on the MMSE were added as further covariate in the model run in the whole cohort.

RESULTS

Correlation models

The three groups are characterized in Table 1. No significant difference was found among the three groups in the size of the VTA ratio, RN ratio, or SN ratio. In the entire cohort, the hippocampal ratio was significantly associated with the VTA ratio ($rho_{(n = 110)} = 0.482$, $p = 9.88e{-}08$, Fig. 3a; *education and MMSE-corrected* $rho_{(df = 106)} = 0.427$, $p = 4.00e{-}06$). This was replicated only in the groups of healthy controls ($rho_{(n = 51)} = 0.586$, $p = 6.00e{-}06$, Fig. 4a; *education and MMSE-corrected* $rho_{(df = 47)} = 0.415$, $p = 0.003$). In the entire cohort the association between the VTA ratio and the memory index was significant ($rho_{(n = 110)} = 0.290$, $p = 0.002$, Fig. 3c; *education-corrected* $rho_{(df = 107)} = 0.291$, $p = 0.002$), while neither the other ratios nor the performance on the Letter Fluency test showed significant associations (Fig. 3b, d, f). Focusing on each diagnostic group, no significant associations were found in the two patient groups (Fig. 4b, c, e, f). Similarly, no effect emerged after the MCI group was separated into amnestic and non-amnestic patients. In the group of healthy adults, memory scores (but not Letter Fluency scores) correlated with the SN ratio ($rho_{(n = 51)} = 0.428$, $p = 0.002$; *education-corrected* $rho_{(df = 48)} = 0.380$, $p = 0.007$), but the association with the VTA ratio was far stronger ($rho_{(n = 51)} = 0.495$, $p = 2.25e{-}04$, Fig. 4g; *education-corrected* $rho_{(df = 48)} = 0.474$, $p = 0.001$). To characterize the role of encoding and retrieval mechanisms in this pattern of findings, the analyses were then re-run separately for z scores derived separately for immediate and delayed recall. The only associations which survived the $p < 0.005$ statistical threshold were those between immediate recall and VTA ratio in the entire cohort ($rho_{(n = 110)} = 0.294$, $p = 0.002$, Fig. 3e; *education-corrected* $rho_{(df = 107)} = 0.296$, $p = 0.002$) and in the group of healthy controls ($rho_{(n = 51)} = 0.483$, $p = 3.33e{-}04$, Fig. 4j; *education-corrected* $rho_{(df = 48)} = 0.460$, $p = 0.001$). The association between VTA ratio and delayed recall only approached statistical significance.

Structural covariance of the VTA

The structural covariance of the VTA extended to hippocampus, insula, and medial prefrontal cortex. The structural covariance of RN and SN was instead regionally confined to the nuclei themselves (Fig. 5).

Functional connectivity of the VTA

In the whole cohort hippocampal volume (Fig. 6a) and memory performance (Fig. 6b) were associated

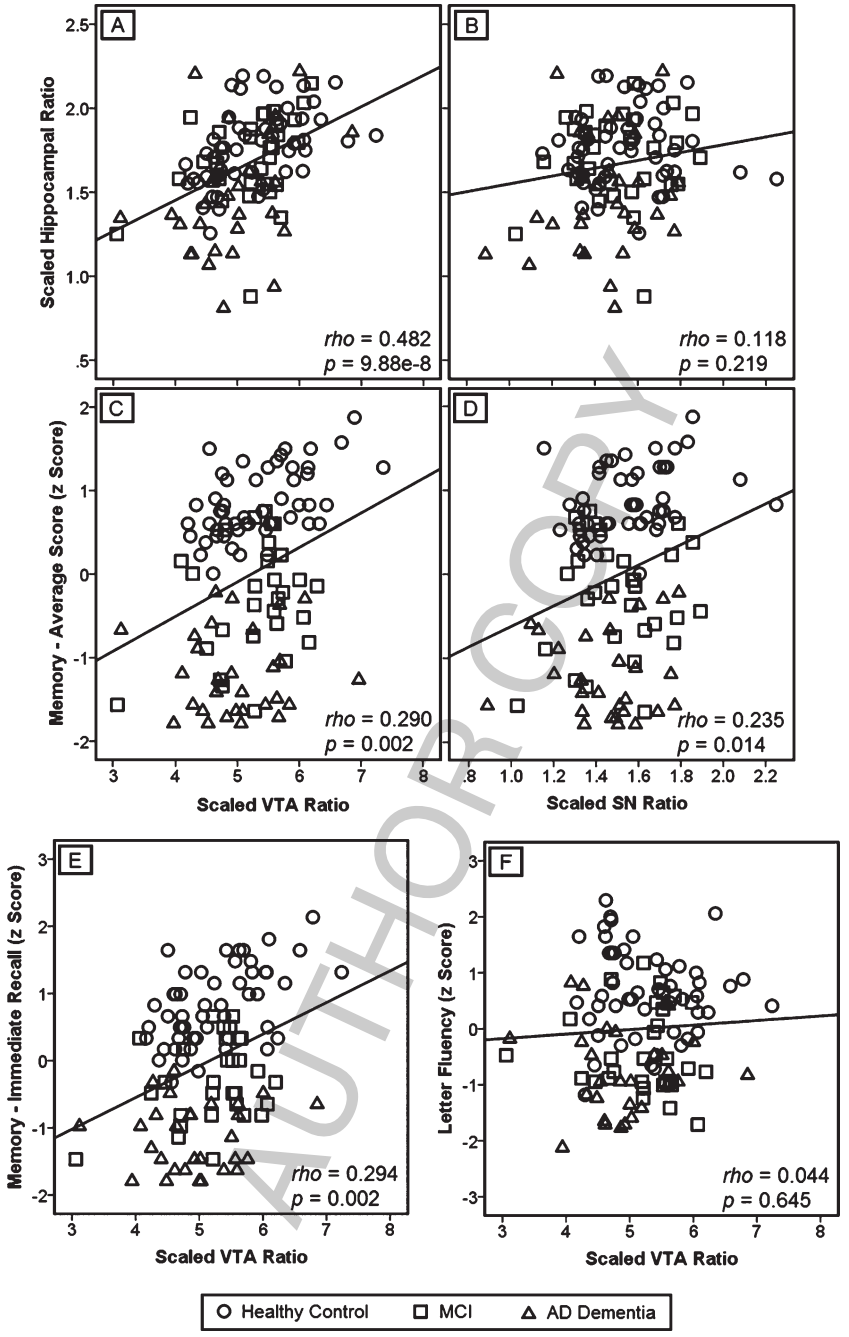

Fig. 3. The linear association models carried out in the entire cohort: between the hippocampal ratio and VTA ratio (a), between the hippocampal ratio and SN ratio (b), between the scores on the Prose Memory test (average of immediate and delayed recall) and VTA ratio (c), between the scores on the Prose Memory test (average of immediate and delayed recall) and SN ratio (d), between immediate recall scores and VTA ratio (e), and between the scores on the Letter Fluency test and VTA ratio (f). Although the figure illustrates linear associations, non-linear associations were run as part of the methodology. Ratios were scaled up (multiplied by 10^3). Models testing the association between RN ratio and clinical indices of AD are not shown.

with the functional connectivity between the VTA and the left hippocampus. Memory performance was also associated with the functional connectivity between the VTA and the medial prefrontal cortex. The association was very similar when immediate and delayed recall were used as predictors.

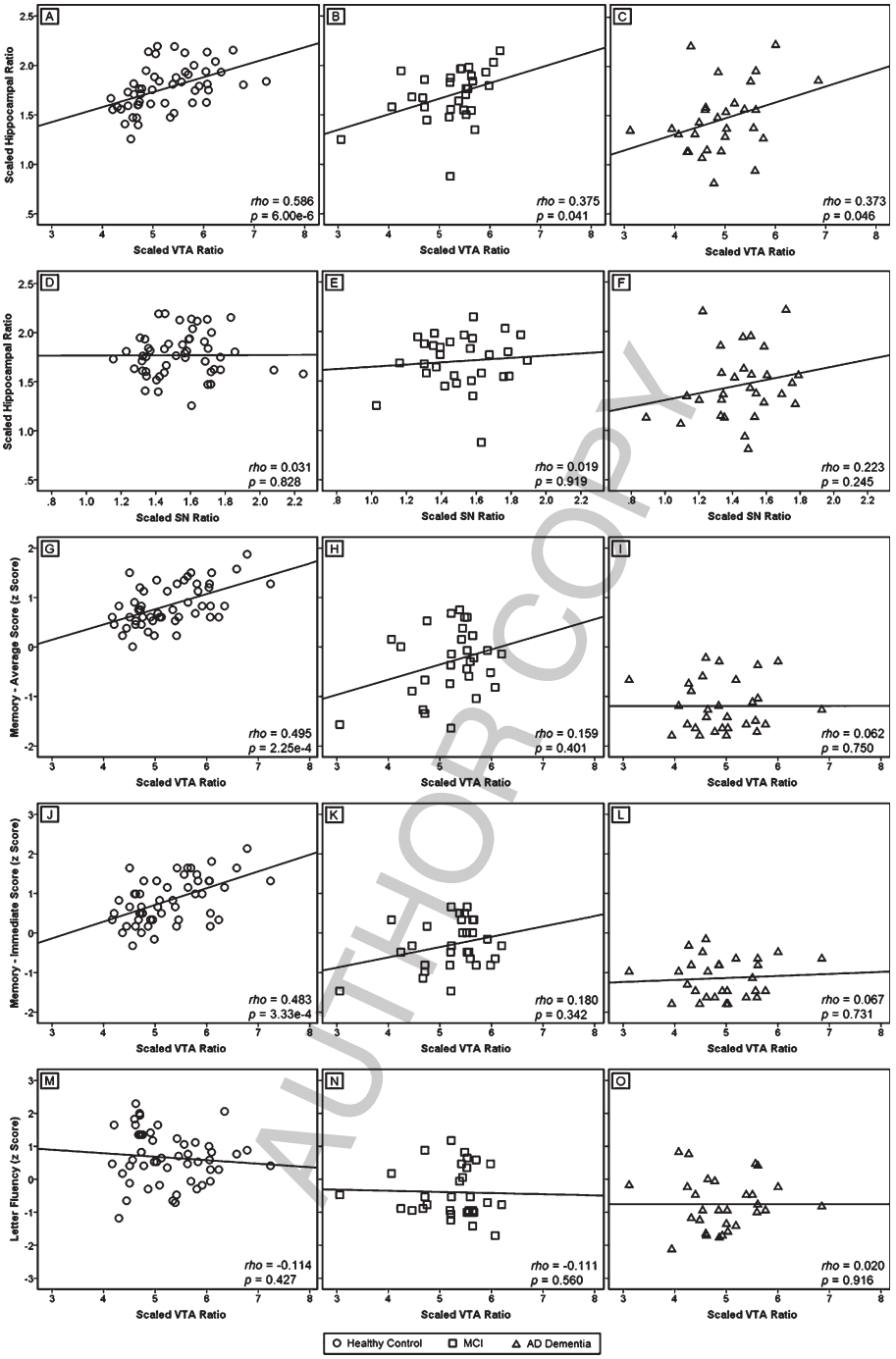

Fig. 4. The linear association models between the VTA ratio and hippocampal ratio in the group of healthy controls (a), MCI patients (b), and patients with dementia (c). This is followed by the linear association between the SN ratio and hippocampal ratio in the group of healthy controls (d), MCI patients (e), and patients with dementia (f). Immediately below, the linear association between the VTA ratio and scores on the Prose Memory test (average of immediate and delayed recall) in the group of healthy controls (g), MCI patients (h), and patients with dementia (i), and, specifically, between immediate recall and VTA ratio in the group of healthy controls (j), MCI patients (k), and patients with dementia (l). The linear association between the VTA ratio and scores on the Letter Fluency test is shown at the bottom in the group of healthy controls (m), MCI patients (n), and patients with dementia (o). Although the figure illustrates linear associations, non-linear associations were run as part of the methodology. Ratios were scaled up (multiplied by 10^3). Models testing the association between RN ratio and clinical indices of AD are not shown.

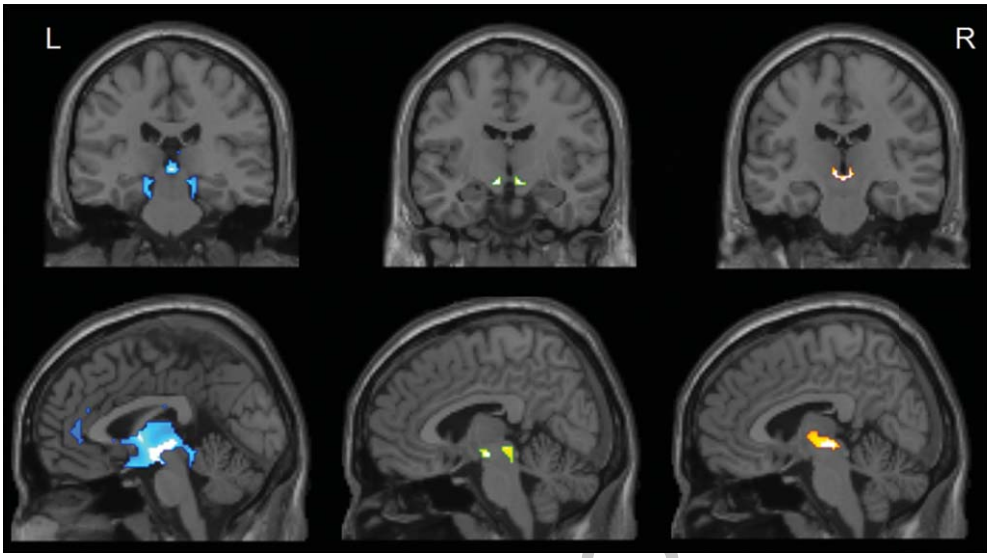

Fig. 5. Structural covariance of the VTA (blue, Montreal Neurological Institute coordinates: $y = -26$, $x = 0$), SN (green, Montreal Neurological Institute coordinates: $y = -10$, $x = 4$) and RN (orange, Montreal Neurological Institute coordinates $y = -20$, $x = 4$). These findings survive a Family Wise Error corrected $p < 0.001$. For color coded regions, please refer to the online version.

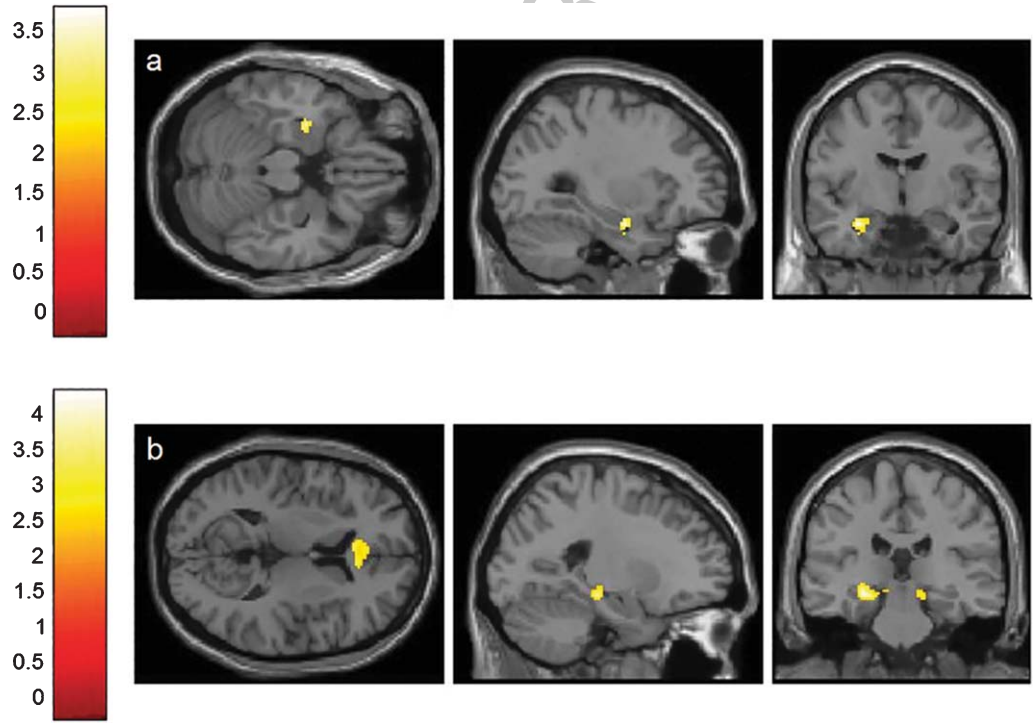

Fig. 6. Functional connectivity of the VTA as a function of hippocampal volume (a). Montreal Neurological Institute coordinates: $z = -22$, $x = -29$, $y = -6$) and memory performance (b). Montreal Neurological Institute coordinates: $z = 9$, $x = -23$, $y = -26$). These findings are significant with an uncorrected $p < 0.01$.

Albeit the analysis of the subgroup of healthy controls revealed a set of trends qualitatively similar to those of the global analyses, the findings emerging from the analyses limited to each diagnostic group did not reach any statistical significance.

DISCUSSION

The findings of this study provide confirmatory evidence from humans in support of a significant role of the VTA in the preclinical phase of the sporadic form of AD, specifically in predicting variability of the typical neurocognitive features of the disease, i.e., hippocampal size and memory ability. Strong correlations were found in the group of healthy individuals, but not in MCI or AD dementia patients. This is in line with the evidence of VTA neuronal loss occurring very early along the disease timeline. In fact, it is expected that only in an asymptomatic population there will be sufficient variability to enable the significant associations to emerge. No similar association was found between the SN ratio and the hippocampus, indicating that it is not a generic volumetric decrease of dopaminergic nuclei associated with hippocampal reduction but, rather, a specific involvement of the VTA in the preclinical stage of AD, as originally found in a murine model [6]. This confirmatory evidence of a selective involvement of the VTA is particularly important because research strives for the detection of a preclinical marker of sporadic AD and novel paradigms of investigation are needed [20].

To contextualize the findings of this study in a manner that can be functionally relevant (i.e., what exact cognitive function is sustained by the VTA and, thus, could be potentially exploited for a preclinical diagnosis of AD), it is informative to address first the anatomy of this region and the connections it forms. The VTA is a group of heterogeneous mesencephalic nuclei located in the midbrain, 163 mm^3 large in humans, and counting about 690,000 neurons [21]. The major component ($\approx$55%) are dopaminergic cells [22], while the remaining part is mainly composed by GABAergic neurons that serve as regulatory inhibitory control [23]. The dopaminergic neurons of the VTA project directly to a series of regions, including the nucleus accumbens, amygdala, hippocampus, and the medial prefrontal cortex [24]. These connections are at the basis of the role of the VTA as a circuital hub in support of a number of functions, e.g., reward mechanisms [25] and emotional processing [26], and in behavioral and psychiatric disorders in which deficits of these functions are a central trait, such as in schizophrenia [27] or craving behavior [15]. In AD, however, it is the depletion of dopaminergic innervation to the hippocampus that is the major pathological change [6]. This finds confirmation in the results of this study. In fact, the anatomical variability of this region was found to be profoundly linked to distinctive features of AD widely and routinely implemented in clinical settings, i.e., the smaller the VTA, the worse these indices.

In human participants, the VTA-hippocampus interplay is normally visible *via* the analysis of functional connectivity [28, 29]. Based on this, if preclinical AD caused neuronal loss in the VTA, the VTA-hippocampus functional pathways should suffer considerably and would be quantifiable *via* measures of hemodynamic connectivity. This would also be in line with the early histological conceptualizations of AD, described as a syndrome that isolates the hippocampal formation computationally [30]. Our findings confirmed this hypothesis, since the functional connectivity between the VTA and the left hippocampus was associated with both hippocampal size and memory performance. It has to be acknowledged that we could not replicate this finding in the group of healthy controls, but, in all likelihood, this was due to a marked decrease in statistical power consequential to the reduction of sample size. Memory performance was also associated with the functional connectivity between the VTA and the medial prefrontal cortex. The medial prefrontal cortex is one of the regions that receives dopaminergic innervation from the VTA [24]. This is also consistent with the role of this structure in long-term memory processes [31].

To put these findings even more in context, it is important to review the recent literature on VTA research. As outlined in the following section, not only does the evidence collected in recent studies support our findings, but it also suggests that the computational role of the VTA-hippocampus pathway appears to be particularly relevant for pathological and clinical processes of early-stage AD. On one hand, in fact, findings indicate that, in the initial stages of disease, neuronal loss in subcortical nuclei is at least as intense as in the mediotemporal complex [32]. This may account for the significant structural covariance we found between the VTA and the hippocampal formation (Fig. 4). On the other hand, convergent evidence associated with the role of the VTA-hippocampus loop is suggestive of a specific cognitive component that could be the key aspect

for a preclinical diagnosis. The computational role of the VTA-hippocampus pathway, in fact, seems to be particularly relevant for material that is associated with a degree of novelty [33]. Specifically, evidence has shown that the VTA-hippocampus interaction is somehow involved in the encoding phase of memory [34]. The encoding phase of mnestic processes consists of the exposure to and acquisition of new stimuli [35]. Obviously, it is widely established that the role of declarative memory impairment is central in AD when memory is intended as a global function (without separating the mechanisms of encoding, retrieval, and storage). However the identification of a memory-related mechanism of clinical relevance during the preclinical stage is a hard task, as this stage is asymptomatic. Longitudinal evidence indicates that, among healthy adults, measures of episodic memory are the best predictors of subsequent conversion to the early symptomatic stage of AD [36]. The simple retrospective use of neuropsychological tests, however, does not allow a clear separation between encoding and retrieval efficiency. It is generally established, however, that measures of immediate recall of a short story (which constitute the Prose Memory test) rely more on encoding than retrieval, as opposed to measures of delayed recall that rely more on retrieval. We thus explored the association between VTA ratio and immediate and delayed recall performance, separately. The findings indicate that immediate recall was the only score significantly associated with the VTA. This corroborates the role of encoding as pivotal cognitive indicator of early VTA disruption. The neural system supporting memory encoding has been characterized with fMRI paradigms, since these allow a temporal separation of the distinct memory phases. Under normal conditions, the hippocampal and perirhinal cortices play a major role during episodic encoding [37, 38]. A recent fMRI study characterized the profile of AD pathology associated with abnormal encoding (de-activation preceding hits and increased activation preceding false alarms) in healthy elderly adults, potentially presymptomatic AD individuals. Findings indicated that abnormal encoding was associated with the presence of a Braak stage I/II, as measured with neuromolecular tau imaging [39], indicating that the encoding stage is particularly informative. In this scenario, if perirhinal and hippocampal activation supports encoding, the connectivity between these areas and the VTA has been found to support the consolidation of encoded material [40].

To draw a parallel with the aforementioned description of the role of the VTA in episodic encoding, the role of the VTA in semantic encoding would also be relevant, as semantic material can also be characterized by a degree of novelty, if the experimental paradigm is appropriately designed. With remarkable convergence, the VTA was found to be the center of repeated semantic encoding for novel material [41]. This is unmistakable evidence that a paradigm of episodic memory encoding and semantic memory encoding might be the main neuropsychological candidate for the detection of VTA suffering. The specific involvement of the encoding and consolidation of episodic and semantic material during the preclinical stage of AD is interlaced with two unconfutable facts that help corroborate the role of this specific cognitive function and, in turn, the VTA as prime suspect of preclinical AD. Firstly, patients with AD show particular difficulties with episodic material encoded in recent, rather than remote past [42, 43], as to draw a trait of gradual failure in day-to-day encoding abilities. Secondly, the concept of semantic encoding for novel material resembles profoundly the operations requested by educational and academic activities. In other words, it is through education that a major amount of novel semantic knowledge gets encoded. Albeit novel semantics is encoded and stored throughout the entire life, this process arguably becomes less and less engaged during the course of adulthood. On this note, if the preclinical stage of AD consists of a reduction of semantic encoding abilities, then it is unsurprising that low levels of education are one of the crucial risk factors for developing the disease. This may shed new light on the role of education levels as integral part of the concept of cognitive reserve, a well-established protecting factor for the onset of AD symptoms [44].

Although this multidimensional set of findings converges towards a robust association between the clinical markers of AD and the size of the VTA, it has to be acknowledged that no significant differences were found between the VTA ratio of healthy controls and patients. This may be due to a multitude of reasons. Firstly, since neuronal loss in the VTA is a presymptomatic occurrence, the variability in VTA size should be informative only in the group of healthy controls. Secondly, when genetics is not characterized, groups of asymptomatic controls are necessarily heterogeneous, as they may include healthy adults as well as presymptomatic individuals. As a consequence, the volumetric properties of the VTA may reflect either a nucleus that had not been subjected to any significant neuronal loss, or, viceversa, a degenerated nucleus that has lost a con-

siderable number of neurons in comparison with its premorbid status. On these grounds, a cross-sectional comparison of VTA size yields limitations and would not be a valid source of information. Longitudinal studies would provide complementary insight, and would also shed light on the connection between reduced dopaminergic input to the mediotemporal regions and possible enhanced susceptibility of these regions to the deposition of the distinctive peptidic hallmarks.

The dopaminergic nature of the neural pathways involved in preclinical AD is suggestive of potential intervention routes. These, however, should be designed to target the system with appropriate timing, i.e., when the disease is at the preclinical stage. Vice versa, dopaminergic therapies introduced at the dementia stage are not expected to be effective. Proof of this is the unsatisfactory outcome of dopaminergic trials for the treatment of AD in the form of seligiline [45]. Other monoamine oxidase inhibitor B molecules have been object of research interest for AD for the regulation of dopaminergic activity, alone and in combination with the conventional cholinergic approach [46–48]. More investigations are needed to study these early changes more in detail, and more clinical studies based on a dopaminergic framework of AD are warranted.

In conclusion, the pre-plaque VTA neuronal loss seen in a rodent model of AD [6] finds here confirmatory support in a human cohort. Today, novel approaches to study the preclinical biological changes of AD are urgently needed [20]. The VTA and the VTA-hippocampal loop are hereby outlined and confirmed as potential preclinical markers of AD that deserve to be investigated more in detail. Clinical focus on memory encoding might provide a neuropsychological measure of assistance. In addition, the dopaminergic nature of this circuit might be suggestive of novel and effective early therapeutic avenues.

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Reduced monoaminergic nuclei MRI signal detectable in pre-symptomatic older adults with future memory decline

Annalena Venneri[1,2]✉ & Matteo De Marco[1,2]

Evidence from murine models and human post-mortem studies indicates that monoaminergic nuclei undergo degeneration at the pre-symptomatic stage of Alzheimer's disease (AD). Analysing 129 datasets from the Alzheimer's Disease Neuroimaging Initiative (ADNI) and relying on the Clinical Dementia Rating as group-defining instrument, we hypothesised that the MRI signal of monoaminergic nuclei would be a statistically significant predictor of memory decline in participants initially recruited in ADNI as healthy adults. As opposed to a group of cognitively stable participants, participants developing memory decline had reduced signal in the ventral tegmental area at baseline, before any evidence of functional decline emerged. These findings indicate that monoaminergic degeneration predates the onset of memory decline in an AD-centred initiative, with a crucial involvement of very-early changes of a dopaminergic region. This translates into potential informative avenues for pharmacological treatment of pre-symptomatic AD.

Cerebral accumulation of beta amyloid and neurofibrillary tangles are widely regarded as central pathophysiological processes at the earliest preclinical stages of Alzheimer's disease (AD)[1]. A prominent body of studies, however, has shown that other neural changes predate the onset of amyloidosis and TAU hyperphosphorylation. These changes affect some of the small monoaminergic nuclei located in the brainstem: locus coeruleus (LC)[2,3], dorsal raphe (DR)[4], and ventral tegmental area (VTA)[5,6].

The LC is the first region of the brain showing non-neurofibrillary TAU-related changes before any cortical deposition of beta amyloid or tangle pathology[2]. The neurons of this nucleus "*accumulate aberrant tau species for decades before frank LC cell body degeneration in AD*"[3, page 1]. The transition from Braak Stage 0 to 1 corresponds to a ≈ 8% volume loss in the LC[7]. Structural MRI can capture this volumetric change with a decrease in T1-weighted (T1W) image signal[8]. Similar to LC, hyperphosphorylated TAU-related changes are observed in the DR prior to any tangle deposition detected in the transentorhinal region[4]. No experimental study, however, has yet provided evidence of a significant structural or functional change in this nucleus at the preclinical stage of AD. At present, this link has only been hypothesised[9].

It has been known for several decades that neurofibrillary pathology in the mediotemporal lobe arises following lesions of the VTA, raising the possibility that dopaminergic denervation is a cause of tangle formation[5]. Consistently with this hypothesis, a study run in the Tg2576 mouse model of AD found significant neuronal loss in the VTA at a stage when no Aβ or TAU pathology was yet visible[6]. Importantly, apoptosis of dopaminergic neurons was correlated with a trend of cellular depletion in the hippocampus and reduction in memory performance[6]. In a follow-up study, it was found that the size of the VTA is correlated with hippocampal volume and memory performance in humans as well[10]. These studies indicate that neural properties of LC, DR and VTA may play an informative role in the pre-symptomatic phase of AD and can thus predict the future development of memory decline. On these bases, we formulated an experimental hypothesis to be tested in a cohort of cognitively unimpaired participants recruited as part of an AD-centred initiative and followed up clinically over multiple timepoints. Specifically, we focused on the concept of functional memory decline as assessed by the Clinical Dementia Rating (CDR) scale[11], and we hypothesised that the MRI signal extracted from the three aforementioned monoaminergic nuclei would show significant cross-sectional and longitudinal differences between declining and stable participants.

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Methods

Participants. Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). The ADNI was launched in 2003 as a public–private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial MRI, positron emission tomography, other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. All ADNI participants provided written informed consent, and study protocols were approved by each participating site's institutional review board. All methods were carried out in accordance with the relevant guidelines and regulations. For research governance and compliance with ethical standards and informed consent please consult the ADNI website at www.adni-info.org and associated material. Additional local ethical approval was not required since the ADNI database contains only anonymised data that are publicly available for download.

The ADNI database was consulted to identify participants who had been recruited as healthy adults with no cognitive impairment and later developed functional memory decline at one of the subsequent follow ups. Longitudinal progression on the global score of the CDR scale was used to operationalise decline. The CDR is a 5-score scale developed by the University of Washington (USA) and used to stage AD and other types of dementia[11]. The global CDR score (CDR-GS) is a valid measure to detect longitudinal functional decline[12]. In this study, functional decline was defined as a CDR-GS increasing from 0 to 0.5 at one of the follow ups. Based on the standard scoring algorithm, memory is the most important CDR category, and is central to CDR-GS scoring. For this reason, functional memory decline is the main drive of a CDR-GS increase from 0 to 0.5. A memory CDR = 0 corresponds to "*no memory loss or slight inconsistent forgetfulness*" while progression to 0.5 tracks the onset of "*consistent slight forgetfulness; partial recollection of events; 'benign' forgetfulness*".

To rule out the effect of simple fluctuations, individual follow-up profiles were inspected to draw a precise CDR-GS trend for each participant. Participants showing a fluctuating trend (i.e., CDR-GS = 0 increasing to 0.5 and then shifting back to 0) were not included in the main study, but were analysed as a separate group. The outcome of this selection was a sample of participants with no initial deficits progressing to functional memory decline devoid of any documented fluctuations ($n = 76$). Two timepoints were defined for each of these participants: a "Timepoint-1" representing the last CDR-GS = 0 measurement with an available MRI image before conversion to a CD-GS = 0.5 and a "Timepoint-2" representing the first CDR-GS = 0.5 assessment with an available MRI image. Since the entire ADNI enterprise is split in four separate sub-initiatives that differ from one another in their protocol of MRI acquisition and in scanner field strength, only datasets acquired entirely (i.e., at both timepoints) as part of ADNI-2 were retained ($n = 43$). An ADNI-2 sample of 86 healthy research participants with CDR-GS = 0 stable for at least 4 years was selected as control group (Fig. 1). As part of the procedures carried out by ADNI, all participants provided their informed written consent.

MRI specifications and processing. Three-dimensional T1W MRI images were extracted for each participant. These included two images acquired in correspondence with Timepoint-1 (CDR-GS = 0 for all participants) and Timepoint-2 (CDR-GS = 0.5 for declining participants; CDR-GS = 0 for stable participants). The specifications of ADNI-2 T1W scans were as follows: 3 T magnetic field, 8-channel head coil, repetition time 400 ms, flip angle 11°, resolution 256 × 256, field of view 26 cm and slice thickness 1.2 mm). Each image was carefully inspected to rule out signal artefacts. The characteristics of the final sample are illustrated in Table 1.

All MRI images were processed using Matlab (Mathworks Inc., UK) and Statistical Parametric Mapping 12 (Wellcome Centre for Human Neuroimaging, London, UK). Each image was segmented to extract the map of grey matter that was then smoothed with an 8 mm full-width at half maximum Gaussian kernel. By doing so, the numerical information associated with each voxel represented the amount of grey matter found in the region encircling the voxel[13]. Additionally, global volumes of grey matter, white matter, and cerebrospinal fluid were quantified using the "get_totals" command line (https://www0.cs.ucl.ac.uk/staff/g.ridgway/vbm/get_totals.m) in Matlab for the calculation of total intracranial volumes.

AD biomarkers. Cerebrospinal fluid biomarkers were available from ADNI for 103 of 124 participants (24 and 79 from the groups of declining and stable participants, respectively) at a temporal distance of < 1 year from Timepoint-1 MRI. Hippocampal volumes were also extracted. These were calculated with an automated procedure that makes use of multiple templates[14], and available at https://niftyweb.cs.ucl.ac.uk/.

Signal extraction from monoaminergic nuclei. Given the small size of the monoaminergic structures of interest, particular care was taken to define a valid and reliable procedure for the extraction, processing and analysis of the T1W-based signal from each nucleus. A binary mask was created for each monoaminergic nucleus of interest (LC, DR and VTA) and for two control regions, i.e., the substantia nigra and the nucleus basalis of Meynert. The definition of all five nuclei was carried out with a methodology informed by the procedures detailed in a number of peer-reviewed publications: DR signal was extracted from a 32 mm^3 ROI centred at x = 0, y = − 27, z = − 9, Montreal Neurological Institute coordinates, as described in previous studies[15–17]. The probabilistic template, calculated by Keren and colleagues[18], was used to define the LC as done in previous research[19,20]. The VTA was drawn as a sphere of 3-mm radius centred at x = 0, y = − 16, z = − 7 following the procedure implemented in previous studies[10,21,22].

The substantia nigra was selected as a monoaminergic control region. In fact, this nucleus is mostly susceptible to aggregation of alpha-synuclein and linked to the presence of motor rather than cognitive symptoms[23]. This nucleus was defined from the Brodmann atlas available on the WFU Pickatlas toolbox[24], following the procedure successfully validated by previous publications[25–27]. A second, non-monoaminergic nucleus was also included in the analyses as further methodological control. The nucleus basalis of Meynert was defined based

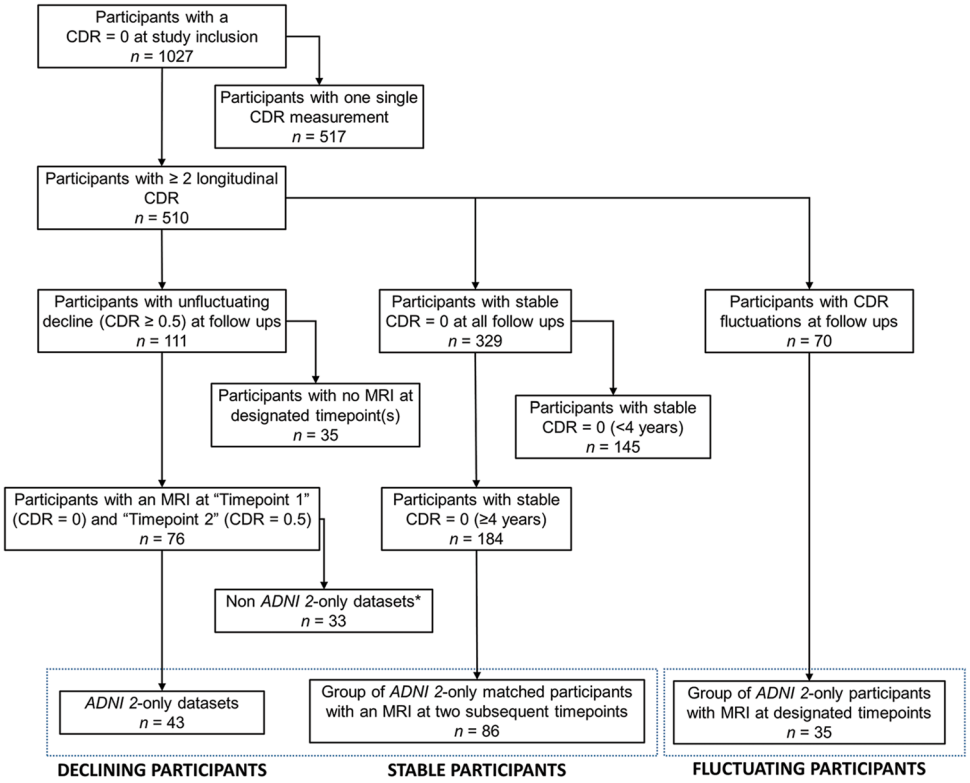

Figure 1. Flowchart showing the procedure of sample selection from the ADNI database. The dotted frames identify the three groups of participants included in the study.

Variable	Stable participants ($n = 86$)	Declining participants ($n = 43$)	Sig. t test
Demographic indices			
Age (years)	75.16 (4.75)	78.15 (6.15)	0.003
Education (years)	16.43 (2.63)	15.93 (2.62)	0.310
Gender (f/m)	49/37	25/18	0.900
APOE genotype (ε2ε2/ε2ε3/ε2ε4/ε3ε3/ε4ε3/ε4ε4)	0/8/0/51/24/3	0/2/1/27/12/1	0.561
Timepoints-1-2 distance (days)	480.07 (201.16)	491.02 (252.29)	0.805
Total intracranial volume (ml)	1487.20 (149.81)	1491.61 (157.68)	0.877
Cerebrospinal fluid AD biomarkers			
Beta Amyloid$_{1-42}$ (pg/ml)	1412.81 (600.21)	1049.72 (616.56)	0.011
Total TAU (pg/ml)	238.60 (87.05)	271.69 (126.96)	0.242
Phosphorylated TAU (pg/ml)	21.80 (8.70)	26.20 (14.20)	0.161
Global neurostructural indices – Timepoint-1			
Grey matter volume (ml)	622.55 (62.25)	583.17 (74.68)	0.002
White matter volume (ml)	405.75 (54.57)	386.77 (59.21)	0.073
Left hippocampal volume (ml)	2.61 (0.31)	2.37 (0.40)	< 0.001
Right hippocampal volume (ml)	2.69 (0.32)	2.44 (0.43)	< 0.001
Global neurostructural indices – Timepoint-2			
Grey matter volume (ml)	612.56 (62.78)	569.82 (74.62)	< 0.001
White matter volume (ml)	402.85 (53.86)	384.93 (59.34)	0.088
Left hippocampal volume (ml)	2.58 (0.31)	2.31 (0.38)	< 0.001
Right hippocampal volume (ml)	2.65 (0.33)	2.38 (0.43)	< 0.001

Table 1. Demographic and global neurostructural indices of the two groups of participants. Cerebrospinal fluid analyses were run on 103 out of 129 participants.

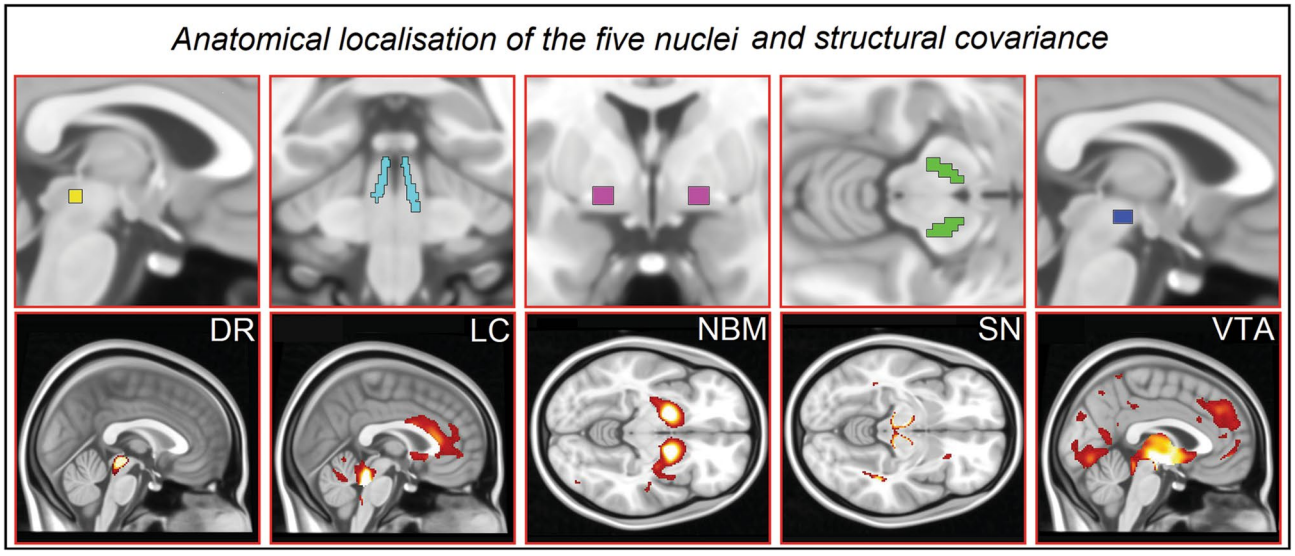

Figure 2. Subcortical nuclei investigated in this study and their structural covariance (in alphabetical order), superimposed to the standard Montreal Neurological Institute space.

on the probabilistic mapping of magnocellular compartments of the basal forebrain[28]. Two spherical masks (3-mm radius each, central coordinates in the Montreal Neurological Institute space: $x = \pm 16$, $y = 0$, $z = -8$) were superimposed to the Ch4 cell group, the subcommissural portion of the basal forebrain that has been historically identified as the nucleus basalis of Meynert[29,30]. All five regions are illustrated in Fig. 2.

The T1W signal intensity was extracted from the binary topography of each nucleus superimposed to the set of smoothed grey matter maps. Additionally, the signal from the pons was also extracted from all scans as correction factor to be used in cross-sectional models. To test for test–retest reliability of signal extracted from each nucleus, a coefficient of correlation was calculated to test the association between the signal calculated at Timepoint-1 and Timepoint-2. These linear associations, shown in Fig. 3, were extremely strong (all r-coefficients > 0.89). Vice versa, coefficients of correlation calculated between nuclei (e.g., DR at Timepoint-1 and VTA at Timepoint-2) were considerably smaller ($r = 0.5$, on average). In addition, the map of structural covariance was calculated for each nucleus, to confirm the appropriateness of the extracted signal (Fig. 2). Furthermore, the signal from 11 pontine regions of comparable size as the VTA (spheres with a 3-mm radius), but fully enclosed within the segmented white-matter map, was also extracted. This served to assess the magnitude of the signal of the experimental nuclei of interest and compare it to the signal of areas devoid of grey matter. The localisation of these areas and the graphical representation of their signal are illustrated in Fig. 4. The signal from the experimental nuclei of interest was significantly stronger compared to these white-matter regions. This strongly supports the genuineness of the measurements and minimises the possibility that the signal included in the analyses was of artefactual nature.

Statistical analysis. *Signal from the nuclei.* Data analysis was run using ISBM SPSS Statistics 23 (https://www.ibm.com/uk-en/analytics/spss-statistics-software). Normality of dependent variables was tested with the *Kolmogorov–Smirnov Goodness-of-fit test*. Since the study was based on cross-sectional as well as longitudinal designs, normality of signal residuals was tested at each timepoint and on a timepoint-to-timepoint subtractive signal change index (Fig. 3f). No breach of the assumption was detected.

To test for signal differences at the two timepoints, one-way *ANOVA*s were run comparing the two groups of participants. Age, the signal from the whole pons and the signal from all eleven pontine regions described in "Signal extraction from monoaminergic nuclei" were used as correction factor.

To test for differences in the longitudinal progression of signal between the two groups, mixed *ANOVA*s were run testing the effect of the 2×2 group-by-timepoint interaction. The temporal distance between timepoints (in days) was added as covariate to these models. A Bonferroni-corrected $p < 0.01$ ($0.05/n$ of tested nuclei) was selected as threshold of significance. Two-tailed hypotheses were tested.

MRI whole-brain. Comparable inferential models as those described in the previous paragraph were devised to analyse T1W images voxel-by-voxel following the procedures of voxel-based morphometry[31]. These analyses were conducted using Statistical Parametric Mapping. Two-sample t-test models were run to compare the two groups at each timepoint, controlling for signal extracted from the pons and from the entire map of grey matter. For these analyses, a standard set-level uncorrected $p < 0.005$ was selected. Only clusters surviving a cluster-level Family-Wise Error corrected $p < 0.05$ were retained as significant. Montreal Neurological Institute coordinates were converted to the Talairach space via a nonlinear transformation (imaging.mrc-cbu.cam.ac.uk/downloads/MNI2tal/mni2tal-m). Spatial coordinate interpretation was drawn using the Talairach Daemon Client[32].

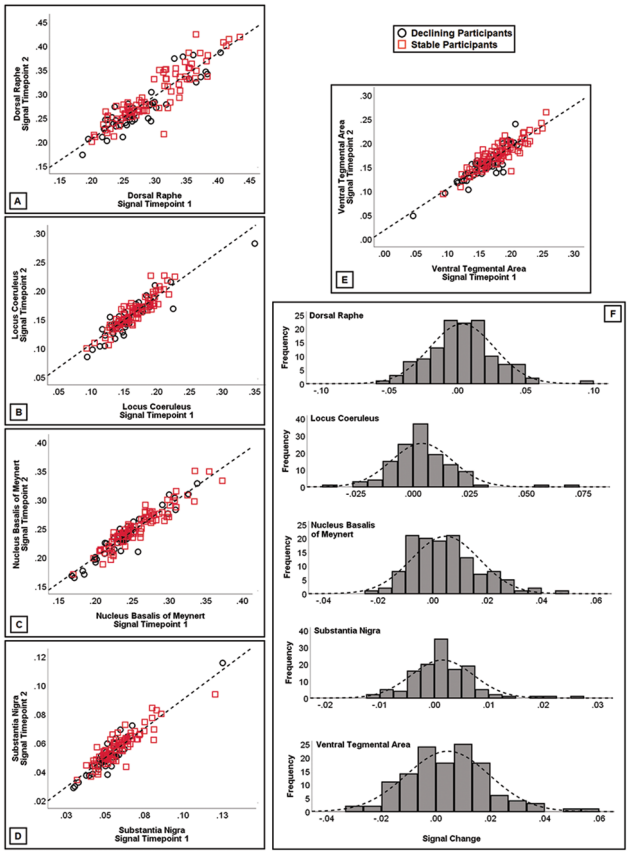

Figure 3. Signal consistency within each nucleus (**A**–**E**) between Timepoint-1 and Timepoint-2. All associations were significant ($r > 0.89$), supporting test–retest reliability of T1-weighted signal from these small regions. A signal change index was calculated subtracting the signal at Timepoint-2 from the signal at Timepoint-1. This index was plotted (F) to confirm normality of data for longitudinal analyses.

Association between nuclei signal and AD biomarkers. The association between nuclei signal and cerebrospinal-fluid AD biomarkers (Beta Amyloid$_{1\text{-}42,}$ total TAU and phosphorylated TAU) was also tested. Partial correlation models were devised. Age, signal from the pons and temporal distance (in days) between MRI and lumbar puncture were used as controlling factors. A Bonferroni-corrected $p < 0.01$ threshold ($0.05/n$ of tested nuclei) was used for these analyses.

Analysis of participants with fluctuating CDR. A group of 35 ADNI-2 participants with a Timepoint-1 CDR = 0, a Timepoint-2 CDR = 0.5 and subsequent reversion to CDR = 0 was also investigated in a series of ancillary analyses. A detailed description of this sample and the results of cross-sectional and longitudinal analyses are reported as Supplementary Material.

Results

The groups of stable and declining participants were matched for education, gender, *APOE* genotype and temporal distance between the two scans, but the group of stable participants was slightly, yet significantly younger and had more grey matter, globally (Table 1). Declining participants had also significantly smaller concentrations of beta amyloid, while there were no differences in TAU levels.

Signal from the nuclei. The outcome of group comparisons at Timepont-1 and Timepoint-2 is shown in Fig. 5a-b. Participants with memory decline had reduced signal in the VTA at both timepoints ($p = 0.002$ and $p < 0.001$, respectively). No difference emerged from the analyses of the other nuclei. The area under the receiver-operating characteristic curve quantifying prediction of decline of the five Timepoint-1 signals is shown in Fig. 6a.

The outcome of the longitudinal analyses is shown in Fig. 5c. No group-by-timepoint interactions were found.

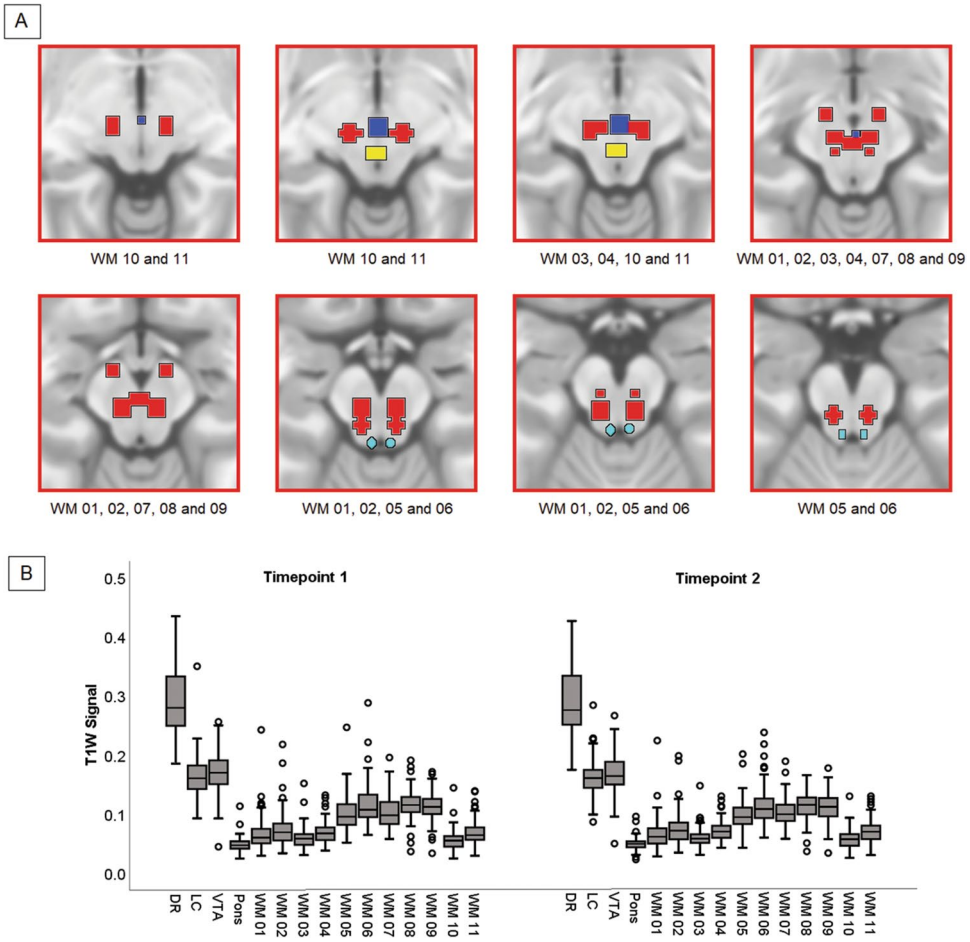

Figure 4. Definition of pontine white matter regions for extraction of T1-weighted signal. (**A**) Eleven spherical regions (3-mm radius) were created around the following Montreal Neurological Institute coordinates. WM 01: x = − 6, y = − 24, z = − 15; WM 02: x = 6, y = − 24, z = − 15; WM 03: x = − 6, y = − 19, z = − 11; WM 04: x = 6, y = − 19, z = − 11; WM 05: x = − 6, y = − 30, z = − 18; WM 06: x = 6, y = − 30, z = − 18; WM 07: x = 0, y = − 21, z = − 13; WM 08: x = − 9, y = − 11, z = − 13; WM 09: x = 9, y = − 11, z = − 13; WM 10: x = − 9, y = − 20, z = − 8; WM 11: x = 9, y = − 20, z = − 8. These regions were created to keep the overlap to a minimum and cover a large portion of the pons. The DR, LC and VTA are indicated in yellow, light blue and blue, respectively. z slices in the Montreal Neurological Institute space are: − 6, − 8. − 10, − 12, − 14, − 16, − 18, − 20. The white-matter regions shown in each slice are indicated below each image. (**B**) The graphical representation of the T1-weighted signal from the three monoaminergic nuclei and the eleven white-matter regions (plus the global pons), showing significant differences in intensity.

MRI whole brain. Cluster-level volumetric differences at Timepoint-1 were limited to the hippocampus, bilaterally (stable participants > declining participant; Fig. 6b), while the difference at Timepoint-2 was much more extensive, covering large portions of the temporal, mediotemporal and limbic lobe (Fig. 6c).

Association with AD biomarkers. No significant association was found between cerebrospinal concentration of biomarkers and the signal in any of the nuclei.

Post-hoc: signal from three years prior to evidence of memory decline. A further MRI measurement acquired ~ 2 to 2.5 years (712–807 days) prior to Timepoint-1 was available for 17 of the 43 declining participants. The signal from all nuclei was extracted at this additional timepoint to characterise further its longitudinal progression. Paired-sample *t*-tests revealed no significant differences between these two timepoints for any of the nuclei.

Post-hoc: follow up CDR measurements in the group of declining participants. Within the group of declining participants, additional ≥ 1 year follow up CDR measurements were available for 27/43 cases. Twelve of these showed evidence of further memory decline leading to dementia (CDR ≥ 1), while the other 15 remained stable at 0.5 at the available follow ups. To test whether differences existed between these two subgroups (suggesting, therefore, potential diagnostic divergences), independent-sample *t* tests (two-tailed) were

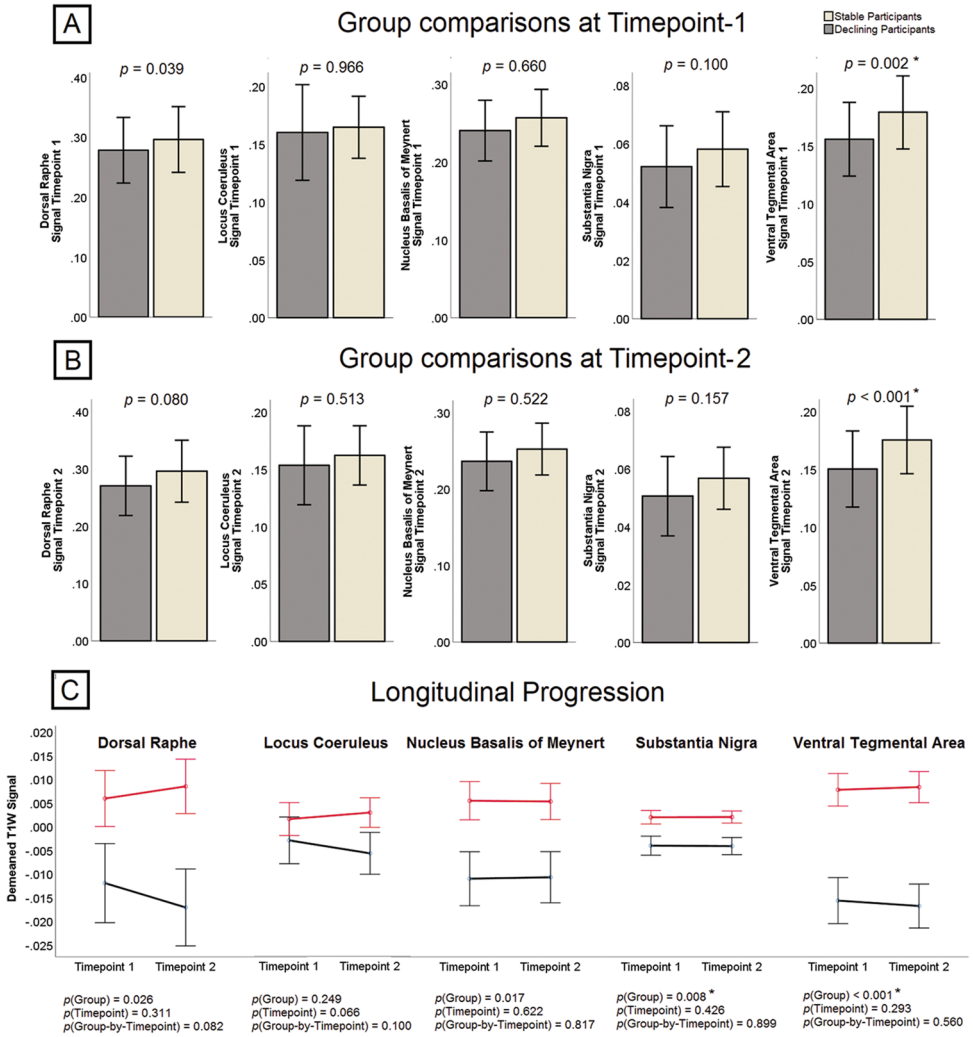

Figure 5. (**A**, **B**) Between-sample comparisons between the group of stable ($n = 86$) and declining ($n = 43$) participants at the two timepoints. Graphs indicate arithmetical means and error bars represent one standard deviation. (**C**) Graphical representation of the mixed *ANOVA* models. Black and red lines show declining and stable participants, and error bars indicate the standard error of the mean. *$p < 0.01$ (Bonferroni-corrected threshold of significance).

run to compare global neurostructural properties and the signal from the five nuclei. No differences were found at either of the two timepoints (all p values > 0.05). The same analyses were rerun as 1 × 3 one-way *ANOVA*s including the third group of 16 participants for whom no CDR follow up was available. Again, no differences emerged (all p values > 0.05), indicating no evidence of aetiological inhomogeneities in the group of declining participants.

Discussion

A body of research indicates that midbrain monoaminergic nuclei undergo significant cellular changes in AD before any significant brain amyloidosis or TAU hyperphosphorylation. To transpose and test this principle in a clinical study, we hypothesised that MRI T1W signal extracted from monoaminergic nuclei would show cross-sectional and longitudinal differences between cognitively unimpaired participants who would later develop memory decline and unimpaired participants remaining cognitively stable. All participants had been recruited as part of an initiative aimed at determining the multi-domain characteristics of the AD spectrum. The results indicate that signal differences in the VTA are already visible at the preclinical stage, before the onset of any memory symptom.

Although cerebrospinal fluid levels of biomarkers were not available for the totality of our sample, the partial sample with biomarkers indicates that, at Timepoint-1, declining participants already showed lower levels of cerebrospinal fluid beta amyloid compared to stable participants.

The two groups showed no differences in the DR or in the LC at either timepoints, but these nuclei showed a trend of longitudinal signal reduction among declining participants ($p = 0.082$ and $p = 0.1$, respectively). Although non significant, these trends are particularly informative for the study of memory decline. In fact,

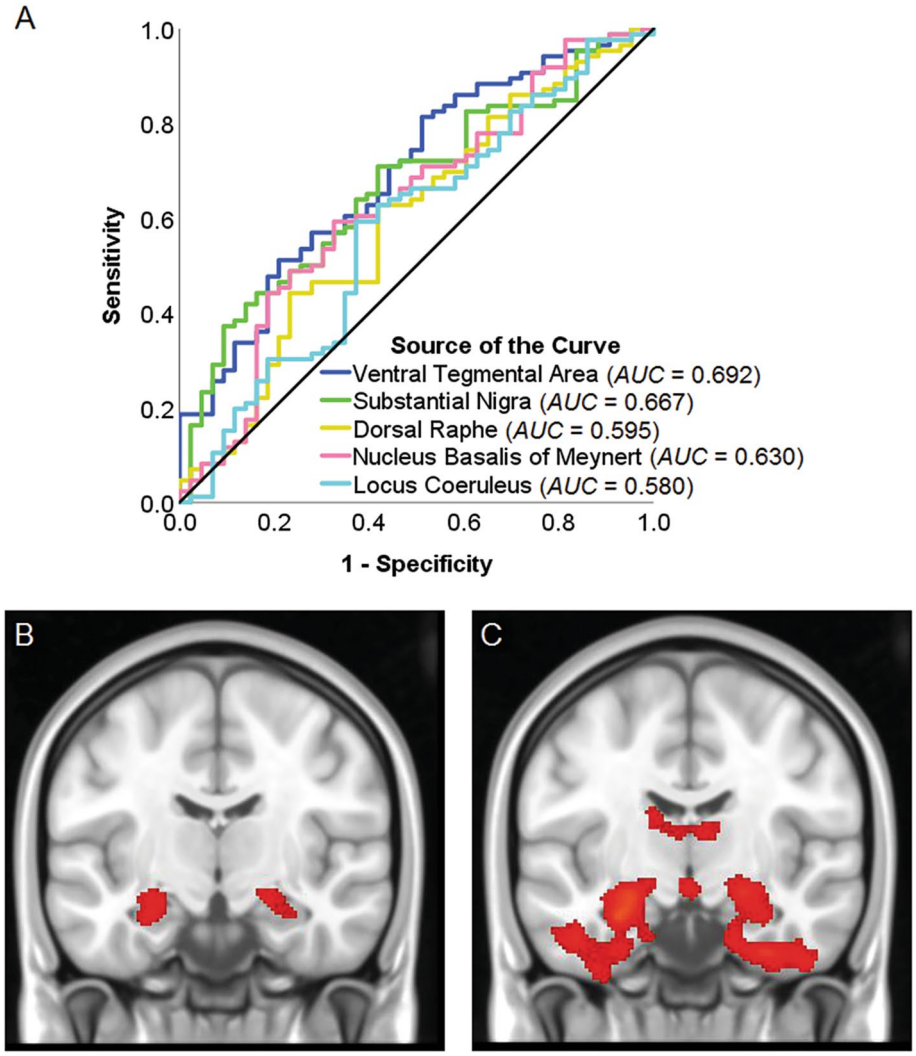

Figure 6. (**A**) Area under the receiver-operating characteristic curve quantifying how well Timepoint-1 monoaminergic nuclei's signal predicts Timepoint-2 group membership. (**B**, **C**) Timepoint-1 and Timepoint-2 (respectively) voxel-by-voxel cluster level differences between the two groups (stable participants > declining participants).

the hippocampus (the region most centrally responsible for episodic memory processing), receives multiple innervations from monoaminergic areas, including axonal projections from the VTA[33], LC[34], and DR[35]. In this context, our findings are consistent with results obtained in murine models[5,6], which highlight the role of early dopaminergic changes as mechanistically involved in hippocampal and memory decline.

The definition of memory decline was based on the increase on the CDR global score (strongly dependent on the CDR memory sub-score). The CDR score is only one of five inclusion criteria for a diagnosis of mild cognitive impairment in ADNI (procedure manuals can be consulted at adni.loni.usc.edu). As a consequence, we cannot equate the CDR increase found in the group of declining participants to a proper progression to mild cognitive impairment. A diagnosis of mild cognitive impairment in ADNI can be made, among others, only in the presence of abnormal performance on the Logical Memory II subscale (from the Wechsler Memory Scale – Revised). This criterion, however, is profoundly dependent on levels of cognitive reserve and needs to be corrected based on levels of education as per procedural guidelines. As a consequence, rather than studying a form of multi-componential decline, we chose to focus on the sole functional decline captured by longitudinal and non-fluctuating CDR increase. In addition, although indirectly important at an interpretational level to help characterise the early stages of AD, these findings do not directly transfer to "preclinical AD", a concept that is based on biological criteria[36]. Longitudinal biomarker data were available only for a proportion of datasets and this prevented us from testing any AD-centred hypothesis in a direct way. The use of the CDR, however, allows one to capture memory difficulties outside of the clinical setting with the additional benefit of the help of an informant. There is evidence, in fact, that individuals may have sufficient resources to sustain a "well-defined" problem such as those typical of laboratory settings, but these may not be sufficient to address "ill-defined"

real-world problems[37]. In the search for early AD biomarkers it is paramount to investigate the contribution that an instrument susceptible to memory changes of minimal intensity such as the CDR may offer.

We also investigated the differences between the two groups of participants by analysing their brain with standard voxel-based morphometry. The results revealed significant differences at both timepoints in associative areas known to be affected in AD, with the hippocampus being the epicentre of the detected differences at Timepoint-1.

This study is not exempt from limitations. First, biomarker data were not bound to the longitudinal MRI timescale, and for this reason we included a single measurement obtained in proximity (< 1 year) of Timepoint-1, with no measurement for Timepoint-2. Second, the methodology was applied retrospectively to MRI images that had not been explicitly collected to highlight small subcortical nuclei. There are other types of anatomical MRI images that can be used to quantify T1W signal from these regions, such as fast low-angle shot sequences[38]. Monoaminergic nuclei are very small and particular care has to be taken when MRI images are used to characterise these regions. For this reason, we ascertained test–retest signal reliability and we adopted a longitudinal design for statistical inference. By doing so, we minimised the chances that the findings could be influenced by artefactual signal variability. Other MRI specifications can, however, be implemented in the future for a more detailed characterisation of the target regions.

In conclusion, we found evidence in support of a statistically predictive link between the VTA and memory decline in a group of cognitively unimpaired participants enrolled in an AD-centred initiative. This confirms and extends previous findings emerged with a cross-sectional design in a cohort of healthy adults[10], and supports the findings of other neuroimaging studies that have highlighted a role of damage in this nucleus in patients with MCI[39]. Dopaminergic neurotransmission may contribute to the pathogenesis of AD[40,41], and further longitudinal investigations carried out in asymptomatic individuals are warranted to characterise the preclinical stage of AD more in detail and thus help devise tailored interventions to be administered at an established preclinical stage. Indeed, there is a large consensus that effective treatment of Alzheimer's disease can only be achieved by a thorough understanding of its causal mechanisms, and that any treatment to be effective has to be started as early as possible. More importantly, it has been suggested that the most effective treatment strategy might be one that is aimed at multiple pathological processes as it is already the case for other chronic diseases[42]. The findings reported in this study contribute to this goal by providing evidence in support of a potential additional early therapeutic target. Although early studies of seligiline in patients with established AD dementia have reported no clinical benefit[43], other monoamine oxidase inhibitors[44,45] and dopamine agonists[46] seem to induce more promising effects, and limited evidence exists in favour of a positive effect of a dopamine agonist in mild cognitive impairment as well[47], warranting a deeper focus on this therapeutic avenue.

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design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: https://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

Author contributions

A.V. contributed design and conception of the study, interpretation of data, revision and finalising of the manuscript; M.D.M. contributed design and conception of the study, data analysis, drafting and revision of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Beyond Episodic Memory: Semantic Processing as Independent Predictor of Hippocampal/Perirhinal Volume in Aging and Mild Cognitive Impairment due to Alzheimer's Disease

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Objective: Given that lexical-semantic decline precedes episodic memory deficits in the Alzheimer's disease (AD) timeline, it is expected that performance on a lexical-semantic task would be associated with mediotemporal volumes independently of the association this region has with episodic memory in the early stage of AD. ***Method:*** Fifty patients with mild cognitive impairment due to AD and 50 healthy adults completed tests of lexical-semantic skills (category fluency test), episodic memory for semantically relevant material (prose memory test), episodic memory for non semantically relevant material (Rey-Osterrieth Figure test), lexical-executive abilities (letter fluency test), and a neurostructural MRI. Hippocampal, perirhinal, entorhinal, temporopolar, and orbitofrontal volumes were extracted. The association between test performance and volume of each region was tested using partial correlations (age-education corrected). The improvement (ΔR^2) in predicting volumetric indices offered by episodic-memory/lexical-semantic processing, once accounting for their counterpart, was tested using hierarchical regressions. ***Results:*** There were no significant findings for control indices. Prose memory accounted for independent portions of volumetric variability within almost all regions. Category fluency accounted for independent portions of volumetric variability of left and right hippocampus and left perirhinal cortex in addition to the predictive strength of the Rey-Osterrieth Figure, and for an independent portion of volumetric variability in the left hippocampus in addition to the predictive strength of prose memory.

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Conclusions: There was an association between hippocampal and perirhinal volume and lexical-semantic processing in addition to the contribution given by episodic memory. This statistical separation supports the importance of lexical-semantic processing as independent indicator of AD.

General Scientific Summary
It is well established that declining semantic skills precede memory symptoms in Alzheimer's disease (AD). Semantic skills are linked to the typical brain regions affected by AD. This link is still present after controlling for episodic memory. Semantic processing abilities can be used to detect preclinical AD.

Keywords: category fluency, mild cognitive impairment, preclinical, perirhinal cortex

It has been widely demonstrated that aspects of memory are dysfunctional in Alzheimer's disease (AD). The latest versions of diagnostic criteria for prodromal and preclinical AD, established by international consensus task forces, are moving toward a view of diagnostic algorithms for early stage AD, in which episodic memory plays a central role (Albert et al., 2011; Dubois et al., 2016). Presence of episodic memory decline, however, is not unequivocally associated with the presence of AD. In fact, it is well known that normal, non pathological aging processes cause some degree of impoverishment of memory skills (Hänninen et al., 1996). Evidence of declining trajectories of memory functions in both types of aging are sometimes not of easy clinical interpretation at an individual level. On this note, a cognitive measure that shows differences in its trajectory of change between the two diagnoses will be of more immediate clinical utility. This has been indicated to be the case for semantic processing. Although it is well established that semantic representations can be accessed via multiple routes, semantic content is often tested exploiting the linguistic modality. As a consequence, semantic processing is commonly (although not exclusively) measured via tests of language assessing lexical variables (Venneri, Jahn-Carta, De Marco, Quaranta, & Marra, 2018).

Although it is well established that deficits in episodic memory are major clinical evidence of the symptomatic stage of the disease, decline of semantic processing and semantic memory occurs in parallel (Barbeau et al., 2012), and there is mounting evidence that it precedes the appearance of episodic memory decline. Subtle insidious changes may occur and might go undetected if not thoroughly assessed, given that for a long time they do not cause any major disruption to everyday cognitive performance. Evidence has repeatedly shown that lexical-semantic impoverishment appears insidiously decades before the onset of clinical symptoms (Amieva et al., 2008; Didic et al., 2011; Le, Lancashire, Hirst, & Jokel, 2011; Snowdon et al., 1996). This might represent an important cognitive aspect to be exploited in a clinical setting for preclinical detection of AD, and confers an advantage to lexical-semantic processing as central diagnostic domain of interest (Papp, Rentz, Orlovsky, Sperling, & Mormino, 2017; Venneri, Mitolo, & De Marco, 2016).

The most common approach to test lexical-semantic processing during neuropsychological assessment of patients is via the category fluency test. Engagement in this test depends upon the integrity of semantic associations, semantic memory, and language (Butters, Granholm, Salmon, Grant, & Wolfe, 1987; Rohrer, Salmon, Wixted, & Paulsen, 1999). The extent of its reliance on executive demands is, however, limited (Henry, Crawford, & Phillips, 2004), as it resembles cognitive operations involved in everyday tasks (e.g., creating a shopping list) normally carried out with little cognitive control (Shao, Janse, Visser, & Meyer, 2014). The mediotemporal involvement in this task has emerged from lesion-mapping studies (Biesbroek et al., 2016), from measures of gray matter density (Venneri, Gorgoglione, et al., 2011) and blood flow that have highlighted the role of the hippocampus and subhippocampal areas (entorhinal and perirhinal cortex) for context-free semantic retrieval, typically requested in the category fluency test (Barbeau et al., 2012). This association has also been confirmed by studies that have measured semantic retrieval via the Delayed Matching to Sample task 48 (DMS-48) visual recognition test (Barbeau et al., 2008; Didic et al., 2010), and via subtests of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) battery (Hirni, Kivisaari, Monsch, & Taylor, 2013). The presence or absence of contextual information is a major feature of declarative memory traces and is functionally related to the anatomical distinction between hippocampal and subhippocampal regions. In fact, episodic memories are characterized by a distinct set of contextual information (i.e., temporal, spatial, and other situational information), whereas semantic memories are not. The hippocampus, at the top of the computational hierarchy of the mediotemporal lobe, is necessary to retrieve context-rich episodic memories, whereas the subhippocampal region, at a secondary level of this hierarchy, is sufficient for the retrieval of context-free semantic memories. (Mishkin, Suzuki, Gadian, & Vargha-Khadem, 1997).

The mediotemporal complex, heavily affected in AD (Braak & Braak, 1991), therefore, appears to be implicated in the support of both episodic memory and semantic processing. Apart from a few investigations (Barbeau et al., 2012; Didic et al., 2011; Joubert et al., 2008), however, the distinction between the two types of declarative memory has not been studied in detail in patients who are at the early stage of AD. Seminal studies carried out on single-patient cases, lesion models, or developmental conditions have indicated that major functional distinctions exist between the hippocampus and the subhippocampal regions (Barbeau et al., 2006; Jonin et al., 2018; Temple & Richardson, 2006; Vargha-Khadem et al., 1997). No study, however, has yet investigated this aspect in AD as a function of a test so widely used worldwide such as the category fluency test. If, on one hand, the mediotemporal/episodic memory link is widely exploited as the main rationale of the criteria for preclinical and prodromal AD (Albert et al., 2011;

Dubois et al., 2016), on the other hand, the mediotemporal and semantic processing link has been relatively understudied. This is surprising, considering that lexical-semantic deficits have been shown to anticipate memory deficits and may thus become the core of novel criteria for preclinical AD (Amieva et al., 2008).

In this study, we focused on the volumetric properties of mediotemporal regions. Because tests of memory are reliant on a multiplicity of cognitive processes, using models of linear statistics, we hypothesized that a measure that relies more on lexical-semantic processing than episodic memory will be a significant predictor of volumetric indices, independently of the predictive strength offered by a measure that relies instead more on episodic memory than lexical-semantic processing (please note that the word *prediction* refers to its statistical meaning). This hypothesis was tested in a cohort of healthy elderly adults and patients with mild cognitive impairment (MCI) due to AD. The regions this study focused on were those affected significantly by AD pathology during the earliest stages, that is, the mediotemporal complex, inclusive of the hippocampus, entorhinal cortex, and perirhinal cortex.

Method

Participants

One hundred participants were included in these analyses. Participants were recruited from the outpatient memory service at IRCCS Fondazione Ospedale San Camillo, Venice, Italy, and included healthy controls ($n = 50$) and patients referred for their first neurological examination for suspected cognitive decline ($n = 50$) who then received a clinical diagnosis of MCI following widely established clinical criteria (Albert et al., 2011; Petersen, 2004) and further corroborated by clinical follow-ups. Each participant had been assessed with an extensive diagnostic protocol, as part of the clinical procedures led by a senior neurologist, including a brain MRI investigation and an extensive neuropsychological assessment. As part of clinical routine, patients received follow-up assessments for confirmation of clinical diagnosis, and conversion to AD was established clinically. Each individual case was assessed and discussed by a team of clinicians including a neuroradiologist, a senior neurologist, and a neuropsychologist. Following consensus among these clinicians, conversion to AD was either confirmed or ruled out. Based on this, inclusion criteria were set as follows: a Mini-Mental State Examination (Folstein, Folstein, & McHugh, 1975) score of 24 or above, and a clinical profile compatible with the presence of potential underlying AD pathology, as determined by a clinical consensus during follow-up examinations. Exclusion criteria were set up to identify and discard any case for whom the etiology was of non neurodegenerative nature (i.e., vascular, traumatic, psychiatric, metabolic; see Winblad et al., 2004). More specifically, these were represented by the presence of an established diagnostic entity of clinical importance (including cerebrovascular or cardiovascular disease at a moderate-severe stage, neuropathy presenting with conduction difficulties, peptic ulcer, sick sinus syndrome), a history of medical events of clinical concerns (e.g., system failure, transient ischemic attacks, brain seizures), the presence of major clinical traits that might represent confounding factors in the study of AD neurodegeneration (i.e., presence of severe neuropsychiatric symptomatology, evidence of abnormal levels of thyroid-stimulating hormone, folates, and Vitamin B12), or specific treatment medication (i.e., memantine/cholinesterase inhibitors consumption at the time of recruitment, or medication for research purposes or with toxic effects to internal organs). Moreover, participants were not included in the study if the MRI images indicated or suggested a major non neurodegenerative problem (e.g., normal-pressure hydrocephalus, previous stroke, brain tumor) that could be associated with the presence of cognitive impairment. Data from neuropsychological assessment at first referral and contemporary MRI scans were used for analyses in this study.

All demographic characteristics are reported in Table 1. Approval for this research was granted by the institutional ethics panel of the IRCCS Fondazione Ospedale San Camillo, Venice, Italy (Reference Number CE: 11.07). Written informed consent was requested and obtained from all recruited participants. The entire research protocol was executed in agreement with the Code of Ethics of the World Medical Association.

Neurocognitive Assessment

A team of experienced clinical neuropsychologists administered an extensive battery of cognitive tests to all study participants. The battery included measures of verbal and non verbal short-term and working memory, visuoconstructive skills, visual long-term memory, verbal long-term memory and new learning, phonological and semantic fluency, visual search and speed of processing, executive functioning, comprehension and receptive language, and verbal and non verbal measures of abstract reasoning. A full description of the instruments is provided in De Marco et al. (2016). The scores obtained on these tests were used as part of the clinical pipeline to establish diagnostic status. For the purpose of this study question, the performance on four tests was further considered:

- The category fluency test was chosen as measure of lexical-semantic processing with no primary reliance on mechanisms of episodic memory. The categories for access to lexicon were car brands, animals, and fruits (1 min per category), as originally chosen for the collection of the Italian normative data (Novelli et al., 1986). Ample evidence indicates that semantic processing is the central cognitive component addressed by this task (Venneri et al., 2018).
- The delayed (10 min) recall of the Rey-Osterrieth Complex Figure test was selected as measure of "non semantic" episodic memory. This test, in fact, consists of encoding and recall of an abstract figure that does not request any semantic processing (Pelati et al., 2011).
- The delayed (10 min) recall of the prose memory test was included as a measure of episodic memory of semantically relevant material. It is well established that semantic processing is used as computational strategy in tests of memory based on verbal material that show a degree of semantic relatedness (e.g., Carlesimo et al., 1998). The trial chosen as part of the prose memory test was the Italian version of the Babcock story (De Renzi, Faglioni, & Ruggerini, 1977).
- The letter fluency test was also included as a measure of linguistic-executive skills. This latter test was chosen as a methodological control because (a) it is methodologically

reliant on a comparable set of technical characteristics as those of the category fluency test (1 min, three trials); and (b) a large proportion of patients with clinically established AD dementia, albeit mostly of mild level, in fact, reach performance levels within normal limits on the letter fluency task (Bizzozero et al., 2013; De Marco, Duzzi, Meneghello, & Venneri, 2017; Herbert, Brookes, Markus, & Morris, 2014). In addition, the neural correlates of this test have been reported to be located mainly in the left frontal lobe, with no overt mediotemporal involvement (Biesbroek et al., 2016; Meinzer et al., 2009). The letters for access to lexicon were F, L and P (1 min per letter), as originally devised for the collection of the Italian norms (Novelli et al., 1986).

All cognitive features are summarized in Table 1.

MRI Acquisition and Processing

A three-dimensional Turbo Field Echo T1-weighted image was acquired on a 1.5 T Philips Achieva scanner as part of a brain MRI protocol (1.1 × 1.1 × 0.6 mm^3 [gap 0.6 mm] voxel resolution, 256 × 256 × 124 matrix size, 250 mm field of view, 7.4 ms repetition time, 3.4 ms echo delay time, and 8° flip angle). This image modality, together with T2-weighted and FLAIR sequences, served to comply with clinical procedures and exclusion criteria. Furthermore, 3D T1-weighted images were also used for modeling and statistical inference. Each image was processed to extract a series of volumetric indices. For this purpose, the standard cortical and subcortical probabilistic segmentation and parcellation procedures of the FreeSurfer Image Analysis Suite (http://surfer.nmr.mgh.harvard.edu/) were implemented. Of the entire output, the volume of the mediotemporal complex was extracted for each participant, maintaining the two hemispheres as separated. The perirhinal cortex was defined as the cytoarchitecturally-defined Brodmann Area 35 (Augustinack et al., 2013), and the entorhinal cortex was defined as the cytoarchitecturally-defined Brodmann Area 28 (Fischl et al., 2009). Both regions are accurately and reliably defined by FreeSurfer, as detailed in their respective publications. The hippocampus was instead extracted from the atlas of subcortical regions (Fischl et al., 2002). Additionally, the volumes of the left and right temporal poles were extracted from the Destrieux atlas (Fischl et al., 2004), as this region is considered an important "amodal" hub that processes similarities among semantic representations (Patterson, Nestor, & Rogers, 2007). Each measure was divided by the total volume of gray matter. By doing so, fractional indices were obtained (Table 1). This was carried out for two reasons: to allow for immediate interindividual comparability and to allow for a simpler interpretation of the hierarchical regression models (see the Statistical Modeling section for details). As a control region not as profoundly affected by AD pathology as the mediotemporal areas, the volume of the lateral orbitofrontal cortex was extracted by conjoining a number of left and right symmetrical orbitofrontal patches (Fischl et al., 2004) and calculating a fractional value. All regions included in this investigation are illustrated in Figure 1.

Statistical Modeling

Analyses were run in the entire cohort and separately in each diagnostic group. To test our experimental hypothesis, three sets of analyses were designed.

Table 1
Characterization of the Sample Included in This Study

Variable	Controls	Patients	Group difference p $U_{\text{Mann-Whitney}}/\chi^2$	
Demographic indices				
Age (years)	69.54 (5.88)	73.86 (6.31)	<.001	
Education (years)	10.94 (4.60)	10.70 (4.33)	.840	
Gender (F/M)	31/19	25/25	.157	
MMSE (score out of 30)	28.98 (1.32)	27.46 (1.92)	<.001	
			p $U_{\text{Mann-Whitney}}$	p $F_{\text{Corrected}}$
Neuropsychological tests				
Letter Fluency test (number of valid entries)	34.74 (12.81)	31.34 (11.08)	.145	.234
Category Fluency test (number of valid entries)	41.36 (9.92)	30.18 (8.66)	<.001	<.001
Prose Memory test—Recall (score out of 25)	13.10 (4.71)	7.32 (4.48)	<.001	<.001
Rey-Osterrieth Complex Figure—Recall (score out of 36)	15.98 (5.66)	8.44 (4.58)	<.001	<.001
			p $U_{\text{Mann-Whitney}}$	
Volumetric measures				
Left hippocampal fraction	.00658 (.00062)	.00595 (.00084)	<.001	
Right hippocampal fraction	.00674 (.00069)	.00605 (.00099)	<.001	
Left perirhinal fraction	.00471 (.00057)	.00431 (.00076)	.012	
Right perirhinal fraction	.00319 (.00052)	.00304 (.00056)	.089	
Left entorhinal fraction	.00331 (.00049)	.00309 (.00059)	.059	
Right entorhinal fraction	.00298 (.00059)	.00282 (.00060)	.124	
Left temporal pole fraction	.00908 (.00089)	.00944 (.00097)	.059	
Right temporal pole fraction	.00924 (.00139)	.00951 (.00127)	.321	
Lateral orbitofrontal fraction	.02302 (.00170)	.02317 (.00149)	.530	

Note. Except for gender ratio, means and standard deviations are indicated. Between-group differences in neuropsychological scores were tested both with uncorrected models and with statistical comparisons corrected for age and years of education. Brain regional fractions were calculated by dividing the regional volume by the total volume of gray matter. F = female; M = male; MMSE = Mini-Mental State Examination.

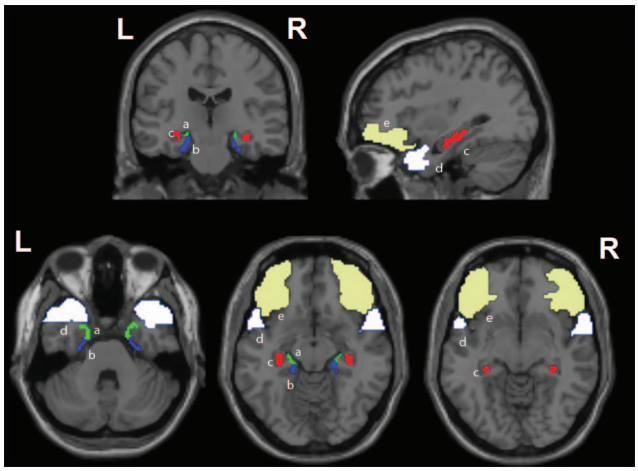

Figure 1. Regions investigated in this study. The hippocampus is shown in red (c) whereas the perirhinal and entorhinal cortices are shown in blue (b) and green (a) respectively. The temporal pole is shown in white (d). The lateral portion of the orbitofrontal region (in yellow, [e].) was chosen as a control area. MNI slices are $y = -22$, $x = 34$, $z = -32$, $z = -12$, and $z = -8$. See the online article for the color version of this figure.

First, a number of group comparisons between patients and controls were run. This served to characterize our sample of patients and verify that the pattern of differences was as expected from a cohort of individuals with a diagnosis of MCI due to AD (Albert et al., 2011).

Second, correlation models were created to test the simple association between the four cognitive tests and volumes of interest. Pearson's *r* correlations were run between each cognitive index and each regional fraction. Control variables added to these models were age and education levels. Age was included because MCI patients were older than healthy controls and also because of its effects on both cognitive performance and brain volumes (Tarroun et al., 2007). Years of education served as control for cognitive reserve (Stern, 2009).

Third, to test our study hypothesis, statistical models were devised to compare the predictive power of the category fluency test as a measure of lexical-semantic processing with that of the Rey-Osterrieth Complex Figure test as a measure of episodic memory, and that of the prose memory test as a measure relying on both episodic memory and semantic processing. The exclusivity with which the performance on each of these three tasks predicted the degree of mediotemporal integrity was modeled with hierarchical multiple regression models, set up for the entire cohort. The scores obtained on two of the three tasks were inserted in a first and second block, respectively, to establish to what degree each test could account for an independent amount of anatomical variability. The *r*-squared (R^2) statistics associated with the combined predictive strength of the pair of tests were extracted for descriptive purposes; *r*-squared change (ΔR^2) statistics were instead inferred to establish the block-to-block predictive improvement. All combinations were inferred for each regional fraction (six regression models, in total). Because fractional indices (and not raw volumes) were used as dependent variables, there was no need to covariate further for a global measure of brain or intracranial volume. In this way, the interpretability of the ΔR^2 statistic was maximized.

Results

Group Comparisons Between Patients and Controls

Between-groups differences were found for the left and right hippocampal fractions and the left perirhinal fraction, with healthy adults having larger structures. No differences were found for the right perirhinal fraction or the entorhinal fractions, nor for the temporal poles (Table 1). Controlling for age did not alter these results. Patients scored significantly worse than healthy controls on the prose memory test, the Rey-Osterrieth Complex Figure test, and the category fluency test. No difference was found for the letter fluency test (Table 1). These findings confirmed that the studied sample had the typical characteristics of prodromal AD.

Association Between Each Ability and Volume of Mediotemporal Structures

In the group of healthy controls, the sole significant association was that between the prose memory test performance and the left perirhinal fraction.

In the group of patients, scores on the category fluency test and prose memory test were both associated with hippocampal size (left and right). In addition, prose memory test scores were also associated in this group with the perirhinal fraction bilaterally and the left entorhinal fraction. No association was found between any mediotemporal fraction and the performance on the Rey-Osterrieth Complex Figure test.

In the whole cohort, the Rey-Osterrieth Complex Figure test was associated with the left hippocampal and perirhinal fraction, and with the entorhinal fraction bilaterally. The prose memory test was instead associated with the hippocampal and perirhinal fraction bilaterally, and with the left entorhinal fraction. The category fluency test, finally, was associated with the hippocampal fraction bilaterally and with the left perirhinal fraction.

Scores on the letter fluency test did not correlate with any fractional measure, neither in one of the diagnostic groups nor across the entire cohort. Similarly, no significant correlation was found in association with the lateral orbitofrontal fraction or with the temporal pole. All *r* scores and *p* values are reported in Table 2.

Semantic Skills Versus Episodic Memory Skills as Predictor of Volume of Mediotemporal Structures

Hierarchical regression models indicated that the scores obtained on the prose memory test accounted for a significant portion of variability of all mediotemporal areas but the right entorhinal cortex, after accounting for the predictive power of the category fluency test or Rey-Osterrieth Complex Figure test. The maximal significant exclusive contribution of the prose memory test ranged between 5.8% and 11.6% of additional variability after accounting for that explained by the category fluency test, and between 4.6% and 13.5% of additional variability after accounting for that explained by the recall of the Rey-Osterrieth Complex Figure test.

Table 2
Coefficients of Partial Correlation Between Mediotemporal Volumes and Measures of Episodic Memory and Lexical-Semantic Processing

Brain region	Healthy controls				MCI patients				Entire cohort			
	ROCF	PM	CF	LF	ROCF	PM	CF	LF	ROCF	PM	CF	LF
Left hippocampal fraction	.005	.207	.141	−.137	.121	**.464*****	**.371****	−.061	**.224***	**.447*****	**.369*****	−.051
Right hippocampal fraction	−.098	.182	.027	−.126	.162	**.473*****	**.442****	−.059	.198	**.440*****	**.356*****	−.051
Left perirhinal fraction	.099	**.412****	.136	.101	.153	**.316***	.190	.080	**.209***	**.400*****	**.234***	.116
Right perirhinal fraction	.035	.270	−.067	−.087	.214	**.311***	.279	−.092	.146	**.288****	.131	−.071
Left entorhinal fraction	.169	.221	.019	.090	.239	**.334***	.243	.098	**.225***	**.301****	.172	.105
Right entorhinal fraction	.183	.085	.006	−.009	.190	.207	.309	−.145	**.227***	.190	.194	−.055
Left temporal pole fraction	.189	.037	−.016	−.260	−.096	−.033	<.001	−.030	−.036	−.074	−.076	−.165
Right temporal pole fraction	−.081	−.066	−.006	−.045	.113	.009	−.034	−.127	−.053	−.075	−.063	−.099
Lateral orbitofrontal fraction	.161	−.116	.051	−.040	−.014	.015	.040	−.148	.035	−.083	.008	−.076

Note. Age and years of education were used as correction factors. Significant ($p < .05$) coefficients of correlations are indicated in bold. ROCF = recall of the Rey-Osterrieth Complex Figure; PM = Prose Memory; CF = Category Fluency; LF = Letter Fluency.
$^{*}p < .05$. $^{**}p < .005$. $^{***}p < .001$.

When the exclusive contribution of the category fluency test was modeled after accounting for the predictive strength of the prose memory test score, a significant block-to-block improvement was found only for the left hippocampus, in which prose memory test scores accounted for 23% of the variability and category fluency scores contributed an additional 3%. When the exclusive contribution of the category fluency test was instead modeled after accounting for the predictive strength of the recall of the Rey-Osterrieth Complex Figure test, a significant block-to-block improvement was found for the left hippocampal fraction (+10% variability), the right hippocampal fraction (+8.6%), and the left perirhinal fraction (+4.5%).

The exclusive contribution of the performance on the Rey-Osterrieth Complex Figure test was not associated with any improvement in the prediction of the volume of mediotemporal structures. The lateral orbitofrontal and temporopolar fractions were not associated with any cognitive test. All results are illustrated in Table 3.

Discussion

In this study, the association between neuropsychological tests commonly used to diagnose cognitive changes induced by early AD and the volume of key mediotemporal regions was investigated in a cohort of healthy controls and patients with MCI due to AD. First and foremost, the prose memory test was confirmed as the task most strongly associated with the entire mediotemporal complex in all models. A second test of episodic memory, the recall of the Rey-Osterrieth Complex Figure, instead showed more modest associations with mediotemporal fractions, and these were limited to the analyses of the entire cohort. Finally, the Category Fluency test was found to be a significant independent predictor of hippocampal and perirhinal volume, even when controlling for measures of episodic memory.

At first glance, these findings may suggest that episodic memory would be the domain most suitable for the study of mediotemporal volumetric properties in healthy elderly adults and patients in the

Table 3
Predictive Exclusivity Shown by the Prose Memory Test (Episodic Memory for Semantically Relevant Material), the Recall of the Rey-Osterrieth Complex Figure (Episodic Memory for non Semantically Relevant Material), and the Category Fluency Test (Lexical-Semantic Processing)

Brain region	Prose Memory		Rey-Osterrieth Complex Figure		Category Fluency	
	CF adjusted	ROCF adjusted	PM adjusted	CF adjusted	PM adjusted	ROCF adjusted
Left hippocampal fraction	**.077** (.083)***	**.135** (.156)****	.008	.018	**.031** (.032)*	**.100** (.111)****
Right hippocampal fraction	**.068** (.073)***	**.121** (.138)****	.005	.013	.024	**.086** (.094)***
Left perirhinal fraction	**.116** (.131)****	**.140** (.163)****	.002	.022	.002	**.045** (.047)*
Right perirhinal fraction	**.065** (.070)**	**.058** (.062)*	.003	.019	<.001	.009
Left entorhinal fraction	**.058** (.062)*	**.046** (.048)*	.013	.034	<.001	.009
Right entorhinal fraction	.007	.006	.025	.025	.010	.009
Left temporal pole fraction	.004	.010	.001	.001	.005	.012
Right temporal pole fraction	.004	.007	<.001	.001	.001	.004
Lateral orbitofrontal fraction	.011	.012	.008	.001	.005	<.001

Note. ΔR^2 statistics are shown, with f^2 effect sizes in parentheses. Significant ΔR^2 statistics are shown in bold. ROCF = Recall of the Rey-Osterrieth Complex Figure; PM = Prose Memory; CF = Category Fluency.
$^{*}p < .05$. $^{**}p < .01$. $^{***}p < .005$. $^{****}p < .001$.

prodromal stages of AD. This is only partially confirmed by our findings. In fact, the performance on the Rey-Osterrieth Complex Figure test (a prominent test of episodic memory) was poorly predictive of mediotemporal volumes. An important issue is clarifying why this was the case. Although the procedures of administration of the prose memory test and the Rey Osterrieth Complex Figure test are, overall, very similar, two major differences exist between these two tests. First, the modality channel (verbal vs. visuospatial) is different. It is well established that visuospatial and verbal-auditory materials are processed via distinct neurophysiological pathways of encoding. Two tests that exploit the same modality may in part share a common portion of variability; vice versa, two tests that exploit different modalities may account for divergent (and complementary) portions of variability. The performance on the category fluency test is commonly aided by visuospatial strategies (Biesbroek et al., 2016; Pakhomov, Eberly, & Knopman, 2016). Yielding information on visuospatial processing, the variability on this task would mitigate the statistical effect outlined by a second visuospatial task, whereas it would not influence the statistical effect outlined by a verbal task. Second, the performance on the prose memory test is known to be influenced by the semantic relatedness of the elements included in the learning material (Carlesimo et al., 1998). The semantic nature of the material the prose memory test depends on is likely to be a crucial factor. This material, in fact, is particularly salient, as it is based on the description of a plot characterized by "the presentation of [a] character, a conflict, an aggravation/complements to the main plot, and a resolution" (Bolognani et al., 2015, p. 138). We argue that the predictive strength shown by the prose memory test and the level by which scores on this task outperformed the level of statistical prediction achieved by the recall of the Rey-Osterrieth Complex Figure might in part be due to the interplay of episodic memory and semantic processing requested by the prose memory task, which is more strongly associated with the integrity of multiple mediotemporal regions. This is also postulated by the model by Mishkin and colleagues (1997), who highlight the hierarchical role of the hippocampal and subhippocampal regions, with the former being crucial for the retrieval of the contextual information normally associated with episodic mnemonic traces and the latter being sufficient for the retrieval of context-free semantic memory. In summary, both differences in modality and semantic content (or a combination of the two) between the recall of the Rey-Osterrieth Complex Figure and the recall of the prose memory test might underlie the pattern of findings described above.

The sole measurement of episodic memory levels for the clinical characterization of patients leads to major methodological limitations in a clinical setting. In fact, tests of this function allow clinicians to make a diagnosis only after episodic memory deficits are present. On the other hand, there is ample evidence indicating that a lexical-semantic decline is present, although subtle, during the preclinical stage of AD, when episodic memory deficits are still absent. This highlights a clinical interval along the disease timeline in which lexical-semantic skills are the only measurable symptom. Considering this as an assumption, and because it has been widely demonstrated that integrity of the mediotemporal lobe is responsible for sustaining lexical-semantic skills (Barbeau et al., 2012; Biesbroek et al., 2016; Didic et al., 2011; Hirni et al., 2013), it derives that there must be some degree of independence in the way that mediotemporal integrity affects episodic memory and lexical-semantic processing. This provides the rationale whereby mediotemporal regions must be associated with lexical-semantic competence in addition to episodic memory competence. As hypothesized, not only did this study produce empirical evidence of an association between performance on the category fluency test and structures of the mediotemporal complex (specifically, hippocampus bilaterally and left perirhinal cortex) - Category Fluency test scores could explain a portion of AD-related neurostructural variability in the left and right hippocampus and, above all, in the left perirhinal cortex independently of that explained by the recall of the Rey-Osterrieth Complex Figure. Moreover, this test significantly improved the predictive strength of prose memory test scores in accounting for the variability of the left hippocampal fraction. Importantly, the absence of any associations between fractional values in mediotemporal complex structures and letter fluency test scores indicates that it is semantic processing, and not any generic lexical processing, that is associated with the mediotemporal lobe.

Experimental findings indicate that, across the life span, lexical-semantic skills remain stable (Park et al., 2002; Verhaeghen, 2003) or show only limited decline (Ferguson, Spencer, Craig, & Colyvas, 2014; Lövdén et al., 2004) of minimal magnitude as opposed to the concurrent decline of episodic memory; vice versa, when lexical-semantic parameters are measured during preclinical AD, they show unequivocal decline, as demonstrated in the Nun Study (Snowdon et al., 1996), by the longitudinal analyses of the linguistic production of well-known writers (Garrard, Maloney, Hodges, & Patterson, 2005; Le et al., 2011; van Velzen & Garrard, 2008), by the analysis of presidential speeches given by former U.S. president Ronald Reagan, diagnosed with AD years after his presidency (Berisha, Wang, LaCross, & Liss, 2015), by other cases of longitudinal evaluations of linguistic abilities across the life span, such as the evidence emerging from the longitudinal analysis of the PAQUID cohort, in which an index of lexical-semantic competence was found to predate diagnosis of AD by 12 years (Amieva et al., 2008), or the analysis of discourse in picture description in pathologically confirmed cases of AD that identified cases 7 to 9 years prior to death (Pekkala et al., 2013). Furthermore, the value of lexical-semantic parameters in predicting conversion to AD has been demonstrated by a clinical study suggesting that a measure of typicality of words could have prognostic valence in MCI (Vita et al., 2014).

Despite accumulating heuristic evidence of the sensitivity of linguistic measures based on lexical-semantic processing to the earliest pathological changes in the disease course, current clinical and research criteria highlight testing of episodic retrieval as the main focus of assessment (Dubois et al., 2016). The goal in clinical assessment, however, needs to be moved to the detection of signs in the preclinical stage or risk stage for any preventative strategy, either through modification of lifestyle factors (Di Marco et al., 2014) or pharmacological treatment (Buckley et al., 2016), to be effective in delaying the dementia stage of the disease. Semantic measures such as the category fluency test, more than any measure of episodic retrieval, can take clinical assessment closer to this goal. The evidence of this study shows that category fluency test performance may represent a proxy of early AD-related pathological changes independent of episodic memory. It is known that the greater sensitivity of measures of lexical-semantic performance depends on their reliance on the integrity of not only the hip-

pocampus but also, most importantly, the perirhinal cortex (Hirni et al., 2013). Models of disease progression estimate that the clinical stage of AD is preceded by a decades-long preclinical phase. In this preclinical phase, changes occur at the pathological level with abnormal regulation of TAU and β-amyloid proteins. In this phase, AD neurofibrillary pathology appears more laterally in the transentorhinal region of the perirhinal cortex (including entorhinal and perirhinal cortex), in what defines Braak Stage I, and only at a later stage does AD pathology spread to the hippocampus (Braak & Braak, 1995). The perirhinal cortex plays a central role within the functional networks supporting retrieval of semantic information - a major requirement for high-level linguistic performance and an important cognitive prerequisite of semantic fluency abilities - and a major computational center for retrieval of context-free memory, of which semantic processing is the main component. Its centrality in lexical-semantic processing makes the perirhinal cortex a likely candidate region for the future definition of a preclinical AD biomarker. The association of category fluency test performance with volumetric values of perirhinal cortex observed in this study suggests that scores on this test might express this structure's level of anatomical and functional integrity. This confirms the findings of other studies that have detected an association between variance in lexical-semantic parameters and volumetric variance in perirhinal cortex in mild AD (Venneri, Gorgoglione, et al., 2011; Venneri et al., 2008). This association was even greater in MCI patients, carriers of the AD-risk gene apolipoprotein ε_4 allele (Venneri, McGeown, et al., 2011). Category fluency test scores may therefore be part of a computational algorithm for a clinical proxy of early AD pathology (Papp et al., 2016) and, as such, show reduced decline among healthy adults free of AD pathology when compared with adults with early encroachment of AD pathology. Indeed, further evidence indicates that the addition of a measure of category fluency performance to a composite cognitive score explains unique variance in amyloid-related decline in amyloid-positive healthy older adults. When these healthy older adults were stratified by neurodegenerative markers, longitudinal assessment showed that those who had more severe neurodegenerative markers had more severe decline in category fluency, suggesting that performance on this test declines very early in the preclinical AD trajectory (Papp et al., 2017). It has to be acknowledged, however, that in our study the association between the size of the left perirhinal cortex and lexical-semantic skills was no longer significant after controlling for performance on an episodic memory test with a semantic load. This finding most likely indicates that by the prodromal stage of the disease, both lexical-semantic abilities and abilities in episodic memory with a semantic load explain most of the structural variance in perirhinal cortex, and the net contribution of each of these functions is no longer distinguishable. In contrast, when a test of episodic memory without any semantic load is used as a covariate, the significant association with perirhinal cortex remains for category fluency.

Recently it was suggested that to achieve the goal of preclinical detection of individuals with early AD pathology, or of individuals at risk of developing AD, a cultural shift had to be made, especially in the framework that describes early AD as a pathological entity limited to the hippocampus, and its primary episodic memory function had to be revised (Barbeau et al., 2004; Didic et al., 2011; Venneri et al., 2016). This is certainly the case at the prodromal, symptomatic stage of the disease, but for earlier detection, a more powerful tool is the evaluation of lexical-semantic skills decline, which more closely mirrors the presence of subtle AD pathology at the prehippocampal, earlier stage of the disease. In addition, this evidence challenges earlier views of AD as an episodic memory disorder with sparing of language, instead suggesting that testing of linguistic semantic skills, and an accurate qualitative analysis of verbal productions, might reveal itself as a better diagnostic tool that can be of assistance within the procedures optimized by clinicians at the preclinical stage. This tool would have the potential for extensive screening of older adults, and, because of its non invasive nature, could become an early and inexpensive biomarker proxy. The evidence of this study supports earlier claims that volumetric measures of the transentorhinal/perirhinal cortex could be a surrogate early marker of AD (Taylor & Probst, 2008), which can easily be obtained in a non invasive way by MRI scanning, and calls for a refinement of imaging protocols, which should focus on quantitative imaging of structures that have the earliest vulnerability to the neuropathological threats of AD. The increasing clinical use of high-field scanners should also contribute to improving imaging protocols of these structures.

One final comment that deserves to be mentioned is the role of mediotemporal areas in a network context. It is widely established that cognitive function is sustained by widespread networks in which individual regions act as computational hubs. Although the default-mode network is the network most distinctively affected by AD pathology (Pasquini et al., 2017; Seeley, Crawford, Zhou, Miller, & Greicius, 2009; Sperling et al., 2009), this involvement likely occurs in a gradual way, following the spread of pathology across Braak stages. On this note, it is not yet understood how changes in volume or thickness of subhippocampal regions are linked to network dysfunction. Published findings indicate significant links between context-free semantic retrieval and network connectivity in the anterior temporal lobe (Gour et al., 2011), but more evidence is needed to assess changes of whole-brain networks in a longitudinal context.

This study is not free from limitations. Although patients were followed up clinically over time, we did not follow up controls. It is possible that some of the controls, healthy at baseline, were actually at the preclinical stage of the disease. We accept this as a possibility that, however, would have little effect at a group level. Furthermore, our procedures did not include a measure of underlying AD pathophysiology. Clinical diagnostic criteria were applied for the identification of patients and controls, and diagnostic status was reached by consensus among clinicians. It is possible that the use of methods such as amyloid imaging or the analysis of cerebrospinal fluid levels of typical peptidic hallmarks of AD may have resulted in the exclusion of a small percentage of the participants. Again, however, this would have had only a mild effect at the group level. Another limitation lies in the multicomponentiality of the prose memory test, which, to some extent, relies on episodic as well as semantic processing. It is in the nature of neuropsychological tests to be sustained by a multitude of cognitive components, of which one represents the core of interest. Based on this, it is not possible to go beyond our speculation that the prose memory test is particularly suitable for tracking down the neuroanatomical modification triggered by AD due to its reliance on an

interplay of episodic memory and semantic processing. Experimental evidence is required to address this point.

In conclusion, the findings of this study indicate that a skill of language (i.e., lexical-semantic processing in the form of retrieval of lexicon following a semantic route) is an ability that is strongly associated with the volume of the mediotemporal lobe. Furthermore, this skill is statistically associated with this region, crucial in AD, in a way that is independent of episodic memory. The category fluency test has good levels of diagnostic classification for AD (Canning, Leach, Stuss, Ngo, & Black, 2004), and our findings support that claim by showing a link with the underlying cerebral structures, harshly affected by AD pathology insidiously and very early in the disease course.

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Age and hippocampal volume predict distinct parts of default mode network activity

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Group comparison studies have established that activity in the posterior part of the default-mode network (DMN) is down-regulated by both normal ageing and Alzheimer's disease (AD). In this study linear regression models were used to disentangle distinctive DMN activity patterns that are more profoundly associated with either normal ageing or a structural marker of neurodegeneration. 312 datasets inclusive of healthy adults and patients were analysed. Days of life at scan (DOL) and hippocampal volume were used as predictors. Group comparisons confirmed a significant association between functional connectivity in the posterior cingulate/retrosplenial cortex and precuneus and both ageing and AD. Fully-corrected regression models revealed that DOL significantly predicted DMN strength in these regions. No such effect, however, was predicted by hippocampal volume. A significant positive association was found between hippocampal volumes and DMN connectivity in the right temporo-parietal junction (TPJ). These results indicate that postero-medial DMN down-regulation may not be specific to neurodegenerative processes but may be more an indication of brain vulnerability to degeneration. The DMN-TPJ disconnection is instead linked to the volumetric properties of the hippocampus, may reflect early-stage regional accumulation of pathology and might be of aid in the clinical detection of abnormal ageing.

The default-mode network (DMN) is a neural pathway that de-activates during overt cognitive processing and activates when one engages in internally-driven mental operations, i.e., conceptual-semantic and "self-projecting" processing like envisioning the future or autobiographical remembering[1,2]. The main set of areas where intrinsic DMN functional connectivity is observed includes the precuneus[3], the posterior cingulate/retrosplenial complex, inferior parietal lobule, medial-prefrontal cortex, lateral temporal cortex and the hippocampal formation[4].

A body of studies has highlighted that both normal ageing and Alzheimer's disease (AD) entail significant down-regulation of the DMN. Gradual down-regulation in DMN functional connectivity is observed from young to late adulthood, and the postero-medial part of the network, including the posterior cingulate/retrosplenial cortex and the precuneus, is one of the regions most influenced by advancing age[5,6]. This holds valid also after accounting for age-associated volumetric decreases[7]. Reduction of connectivity in the posterior portion of the DMN is also observed in healthy adults with amyloid-β burden[8], young carriers of *PSEN1*/*PSEN2*/*APP* mutations[9], patients with a clinical diagnosis of AD[10–12], adults with a diagnosis of amnestic MCI[13,14], and in the continuum from healthy adulthood, to MCI, to AD dementia[15,16].

These findings indicate that the links between the DMN and ageing and between the DMN and AD may be qualitatively not specific. Although the effect of ageing and AD on the posterior part of DMN is qualitatively similar, however, this appears exacerbated in AD[17].

The studies that have investigated the effects of ageing and AD on the DMN were devised based on the statistical comparison (i.e., via a between-sample *t* test) of patients and age-matched healthy adults[11,17,18]. This inferential method centres around group membership as a unique dichotomic independent variable. With this design it is assumed that each measurement can contribute to one group only. At present, however, it is unknown to what extent reduced DMN functional connectivity seen in a single patient is the result of AD, ageing, and/or their interplay. On one hand, no patient with AD is immune to the incidental effects of ageing. At the same time, cognitively healthy adults may have sub-clinical levels of AD pathology. As a result, ageing and AD may interact at the individual level, highlighting a methodological limit in the use of group comparisons.

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Group	Number
I. Healthy Adults aged 21–40 years old	49
II. Healthy Adults aged 41–64 (*) years old	49
III. Healthy Adults aged 65–72 (**) years old	50
IV. Healthy Adults aged 73 or more	43
V. MCI patients with MMSE score > 27	39
VI. MCI patients with MMSE score 24–27	47
VII. Patients with AD Dementia (MMSE < 24)	35

Table 1. Schematic description of the cohort. (*) The separation bar was set at the age of 64 because adults younger than 65 who are diagnosed with AD are referred to as "early-onset" patients. (**) The separation bar was set at the age of 72 because 72.8 years is the healthy life expectancy at birth in Italy, as estimated by the World Health Organization in 2015.

To detect distinctive associations between the DMN and each of the two processes (ageing and AD), therefore, an alternative method based on linear regression was implemented. DMN functional connectivity was modelled as a function of: (1) a variable conceptually more linked to ageing than AD (age); and (2) a variable conceptually more linked to AD neurodegeneration than ageing (focal volume of the hippocampus). The statistical effects of these two predictors were modelled based on the increase in the fit of the model at the inferential level. Additionally, data were also analysed with canonical group comparisons, in order to highlight the different outcomes emerging from the use of distinct inferential methods. We expected that a pattern of statistical associations more specific to the neurodegeneration seen in AD than the outcome of group comparisons would emerge from these analyses.

Material and Methods

Participants. Three-hundred-and-seventy MRI datasets were considered for inclusion. This cohort was recruited between June 2011 and July 2016 in the memory clinic at the IRCCS Fondazione Ospedale San Camillo, in Venice, Italy as part of a project funded by the Italian Ministry of Health led by AV, and includes healthy adults aged between 22 and 85 years old, patients diagnosed with mild cognitive impairment[19], and patients diagnosed with dementia of the AD type[20]. All diagnoses were reached by consensus among clinicians, and were based on a neurological screening, an extensive battery of neuropsychological tests, and, in a proportion of cases, follow-up appointments. A series of exclusion criteria served to rule out non-neurodegenerative aetiologies that might be behind the onset of cognitive symptoms (i.e., psychiatric, metabolic, traumatic or vascular). These criteria were defined as follows: evidence of a significant diagnostic entity, as revealed by MRI, which might account for the presence of cognitive impairment, depressive, anxious or other psychiatric symptoms of clinical relevance, pharmacological treatments with psychotropic medications, with drugs for research purposes or with toxic effects to internal organs, clinically significant diseases other than those consistent with the objective of the study, a previous history of transient ischaemic attacks, a diagnosis of severe vascular pathology with excessive hyperintensity load (quantified with a Fazekas score > 2)[21], presence/diagnosis of uncontrolled seizures, peptic ulcer, sick sinus syndrome, neuropathy with conduction difficulties, significant disabilities, evidence of abnormal baseline levels of folates, vitamin B12 or thyroid-stimulating hormone. Following the application of these criteria, 34 datasets were excluded from the study. Moreover, 7 additional datasets were excluded as clinical history was incomplete and diagnosis uncertain. The remaining 329 MRI datasets were taken forward to the functional MRI (fMRI) preprocessing pipelines and were subjected to a quality check to rule out the presence of technical exclusion criteria. During these operations, 10 datasets were excluded because of signal artefacts affecting the BOLD signal, and 7 datasets were excluded because of excessive motion (see below for details). The final sample included 312 datasets (Table 1).

All participants completed an MRI protocol inclusive of T1-weighted and a resting-state fMRI acquisitions, plus a number of clinical sequences (diffusion-weighted, T2-weighted, and FLAIR), which were reviewed by a senior neuroradiologist to comply with study criteria, as described above. All participants aged 40 years old or older completed, as part of their clinical profiling, an extensive battery of neuropsychological tests to ascertain their clinical status. These included tests of short-term and working memory, episodic memory, lexical-semantic processing, abstract-conceptual reasoning, attentive-executive functions and visuoconstructive abilities. A complete description of the battery can be openly consulted in a previously published article[22]. Cognitive scores were made available for contingent *post hoc* analyses.

Choice of the predictors for the study of ageing and AD. Two variables were chosen based on their association with the two developmental trajectories investigated in this study (ageing and AD). Although no demographic, pathological or clinical variable exists that truly depends on the expression of exclusively one of the two trajectories (i.e., a variable solely linked to ageing, or solely linked to AD), the following two indices emerged as having strong conceptual links "preferentially" with one of the two trajectories.

The "number of days of life at scan" was chosen as a variable more dependent on ageing than AD. This variable is equivalent to and more precise than the typical measurement in years. The "datedif" function in Microsoft Excel was used to calculate the exact number comparing the date of birth and the date of the MRI scan.

Hippocampal volume was instead chosen as a variable more dependent on AD than ageing. The hippocampus is one of the earliest areas affected in AD[23] and is central in the typical presentation of the disease. Evidence of volumetric decrement of this structure is considered an important marker of AD-related neuronal injury[24]. Moreover, this variable is also informative among healthy adults, as hippocampal volume and integrity are predictors of cognitive decline in this population[25]. Finally, this variable offers a major methodological advantage over other potential proxies of AD (e.g., the score on the Mini Mental State Examination), because it is characterised by an ample numerical variability among old as well as young adults[26], and, specifically in this latter sub-population, it is associated with memory performance[27,28]. Moreover, even at an age as young as ≈20 years, genetic variables that are biological modulators of the pathophysiological mechanisms of AD do appear to have an impact on the morphometry of the hippocampus[29,30].

MRI acquisition. Three-dimensional T1-weighted images and resting-state acquisitions were modelled for statistical inference. These sequences were acquired with a Philips Achieva 1.5 T machine as part of a single MRI protocol. Turbo Field Echo T1 images were based on a $1.1 \times 1.1 \times 0.6\,mm^3$ (gap 0.6 mm), voxel resolution, $256 \times 256 \times 124$ matrix size, 250 mm field of view, 7.4 ms repetition time, 3.4 ms echo delay time, and 8° flip angle. Resting state fMRI scans were preceded by 20 s of dummy volumes set to allow the scanner to reach equilibrium. At least 200 volumes were acquired for each participant, each volume consisting of 20 slices acquired axially and contiguously, in ascending order, with the following parameters: $3.28 \times 3.28 \times 6.00\,mm^3$ voxel dimension, 64×64 matrix size, 230 mm field of view, 2 s TR, 50 ms TE and 90° flip angle.

MRI processing. The methodology was carried out using Statistical Parametric Mapping software (SPM) 8 (Wellcome Centre for Human Neuroimaging, London, UK) and Matlab R2011b (Mathworks Inc., UK).

The SPM "new segmentation" tool was used to separate each T1-weighted image into six tissue maps. Of these, the two maps of neural tissue (grey matter and white matter) and the map of cerebrospinal fluid were quantified in volumetric terms using the "*get_totals*" script (www0.cs.ucl.ac.uk/staff/g.ridgway/vbm/get_totals.m). Total intracranial volumes represented the arithmetic summation of the volumetric quantification of the three maps for each participant. The grey-matter ratio was computed by dividing the total grey-matter volume by the total intracranial volume.

The hippocampus was processed using the STEPS procedure, fully automatised and available online (http://cmictig.cs.ucl.ac.uk/niftyweb/). This methodology allows an accurate segmentation of the hippocampus in its native space from T1-weighted images through the exploitation of multiple templates[31]. In order to minimise any effect of lateralisation, the volumes of the left and right hippocampus were averaged.

Resting-state fMRI scans were preprocessed using a standard pipeline that included slice timing, realignment, normalisation, temporal filtering (0.008–0.1 Hz) and a 6-mm smoothing. In-scanner motion parameters were inspected to verify the absence of excessive movement, which could induce artefactual alterations to the fluctuations of the blood oxygen level dependent signal. None of the scans had to show motion larger than the size of 1 voxel in any of the directions. Where excessive movements were located at the very beginning or very end of the functional run, the problematic volumes were removed (and, by doing so, the rhythmicity of neurogenic signal fluctuations was not altered), and the preprocessing pipeline was restarted. This was done for 6 scans, for which at least 180 volumes were retained. When excessive movement was instead located in the middle of the run, the participant was excluded from the study (7 datasets in total, as mentioned above).

A group independent component analysis was set up to extract the DMN[32]. To do so the GIFT toolbox was used (GIFT v1.3i; mialab.mrn.org/software/gift). The Infomax optimisation principle was chosen, and the number of components was set at 20, following the choice made in a very large study of more than one thousand scans[33]. Given the very high inter-rater level of agreement on the identification of the DMN among the output components[34], the map with the spatial characteristics of the DMN was selected based on the judgement of two independent raters.

MRI modelling. Descriptive statistics were run to characterise the distribution of the two proxies, and Pearson's *r* coefficients of correlation were calculated to test for collinearity.

Linear models were devised to predict the variability of DMN connectivity as expressed by *z* scores. In order to replicate the established finding that ageing and AD have an effect on the connectivity of the posterior section of the DMN, *t* tests were devised. A first model was run to compare a sub-group of patients with clinically-established dementia of the AD type (group VII in Table 1) and a sub-group of healthy adults matched for age ($p = 0.457$), education ($p = 0.471$) and gender ($p = 0.878$). A second model was then run to compare a sub-group of young adults (group I in Table 1) and a sub-group of healthy elderly adults (group IV in Table 1). These two group comparisons were run to test for differences ascribable to AD and ageing on the DMN, respectively.

To explore the distinctive predictive effect of the two predictors, uncorrected multiple regression models were initially set up. Subsequently, multiple regression models were run correcting for the homologous proxy (i.e., the predictive effect of age correcting for hippocampal volume, and the predictive effect of hippocampal volume correcting for age). Finally, fully-corrected multiple regression models were run, including the homologous proxy, gender, levels of education (to control for cognitive reserve), total volumes of grey matter (to control for brain reserve), and the ratio of grey matter (to control for global atrophy). These two latter covariates were not correlated with one another ($r = 0.055$, $p = 0.335$). All contrasts were devised in the direction of our hypotheses (i.e., a negative association between network connectivity and age, and positive association between network connectivity and hippocampal volume). The same inferential models were also run to predict the effect of age and hippocampal volume on the whole-brain map of grey matter.

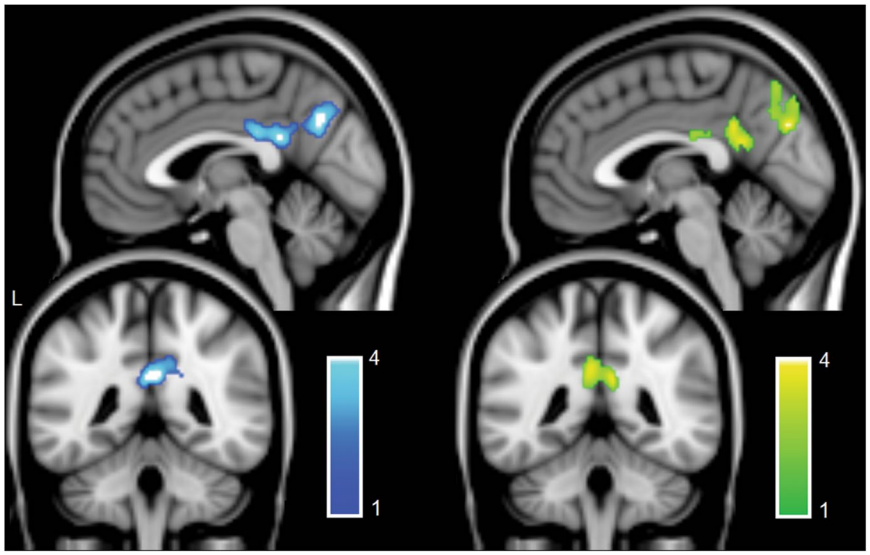

Figure 1. Results of the *t* test models testing the effect of ageing (healthy young > healthy old, cyan overlay) and the effect of AD (healthy old > AD dementia, green overlay) on the DMN. The same pattern emerged from the two models (x = −2; y = −44). *z* scores are indicated on the side of each output.

Ethical approval. All procedures involved in this study were carried out in accordance with institutional ethical standards and with the 1964 Helsinki declaration and its later amendments. This study had received approval by the Institutional Review Board of the IRCCS Fondazione Ospedale San Camillo (Venice, Italy), (Protocol No. 11/09 version 2).

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

Sharing of data. The authors have no permission from participants to share their data beyond the team of collaborators of the principal investigator (AV).

Results

The correlation between the two predictors was significant but was limited to $r = -0.326$, ruling out collinearity issues. The results of the two *t* tests run on sub-groups of the cohort are reported in Fig. 1 and Table 2. Healthy elderly adults had more connectivity than patients with AD dementia in a large postero-medial cluster encompassing Brodmann Area (BA) 7, 23 and 31 (posterior cingulate/retrosplenial cortex and precuneus). Similarly, young adults had more connectivity than elderly adults in the same areas.

The results of the regression models investigating the pattern of distinctiveness for each predictor are illustrated in Fig. 2. A negative association was found between age and functional connectivity of the DMN in a large postero-medial region covering the retrosplenial/posterior cingulate cortex and the precuneus, and in the insula bilaterally. This was partially mitigated after controlling for hippocampal volumes, and still retained its core features in the fully-corrected model (Table 3, Fig. 2b). The uncorrected model revealed a similar (positive) association between hippocampal volumes and DMN connectivity of a large postero-medial region, and a smaller cluster located at the border of the right temporal and parietal lobe (showing its peak in BA 22 but extending also to BA 21, 40, 41, and 42). In the partially-corrected model the postero-medial cluster was largely downsized, and it was no longer significant in the fully-corrected model. On the other hand, the right temporo-parietal cluster retained significance (Fig. 2c). Using the left or right hippocampal volume as proxy instead of the bilateral average resulted in the same pattern of results. Similarly, the inclusion of total intracranial volumes among the covariates (in lieu of the ratio of grey matter) resulted in unaltered findings.

To characterise the role of this temporo-parietal region more in detail, the local DMN signal was extracted. Correlation models were created to test its association with the two independent variables and performance on all cognitive tests. The association between the strength of the DMN in this cluster (expressed as *z* scores) and the two independent variables is illustrated in Fig. 2d. This value was only associated with hippocampal volume, not with age. Moreover, the sole neuropsychological test showing a significant association with regional DMN strength was the recall of the Prose Memory test ($r = 0.158$, $p = 0.009$; Fig. 2e). This association was even more significant after correcting for age and for all covariates included in the main models, and the DMN connectivity in the posterior cingulate cortex ($r = 0.180$; $p = 0.003$).

Discussion

Evidence indicates that the posterior portion of the DMN seems to be particularly susceptible to both the process of normal ageing and the pathological impact of AD. With a first set of analyses, we confirmed these well-established findings: ageing and AD are both associated with decreased functional connectivity of the DMN in the posterior cingulate/retrosplenial cortex and precuneus. These results were obtained with *t* tests comparing

Cluster No.	Cluster *pFWE*	Cluster size (voxels)	*Z* Score at Local Maximum	Side	BA	Region	Talairach Coordinates		
							x	y	z
Healthy Elderly Adults > AD Patients									
1	0.000	1640	4.85	L	7	Cuneus	−6	−72	33
			4.28	R	7	Cuneus	12	−70	33
			4.19	L	7	Cuneus	−12	−68	29
			3.72	L	31	Cingulate Gyrus	−4	−43	30
			3.70	R	7	Precuneus	10	−60	36
			3.68	L	31	Cingulate Gyrus	−10	−29	33
			3.65	L	23	Posterior Cingulate	−4	−47	23
			3.64	R	7	Precuneus	2	−65	51
Young Adults > Healthy Elderly Adults									
1	0.000	1290	4.82	R	31	Cingulate Gyrus	4	−41	26
			4.73	R	23	Cingulate Gyrus	6	−20	27
			4.47	L	7	Precuneus	−2	−62	36
			3.96	R	31	Precuneus	14	−47	34
			3.78	L	23	Cingulate Gyrus	−2	−28	27
			3.54	L	31	Precuneus	−10	−55	30
2	0.003	522	4.31	R		Claustrum	36	14	1
			3.99	R	13	Insula	38	10	−2

Table 2. Effect of Alzheimer's disease degeneration and of the process of ageing on the functional connectivity of the DMN detected by between-sample *t* test models. BA: Brodmann Area; L: Left; R: Right.

groups of participants. A different pattern, however, emerged from the regression models. In these models we focused on the distinctive impact of two variables known to be conceptually more linked either with normal ageing (days of life at scan) or neurodegeneration of the AD type (hippocampal volume). The results indicate that the association between functional connectivity in the posterior cingulate/retrosplenial cortex and precuneus and days of life at scan was significant beyond the statistical prediction offered by hippocampal volume. This does not mean that hippocampal volume does not predict functional connectivity in this region, or in this specific cohort (in fact, we did replicate with t test models the findings reported in the literature), but it means that the numerical variability predicted by hippocampal volume in the postero-medial territory is not significantly more than that predicted by age. This indicates that the typical posterior reductions of functional connectivity seen in the DMN do not seem to be a specific marker of abnormal ageing, but rather represent a general vulnerability of brain physiology to multiple processes, as already proposed[35].

A novel finding of this study is the exclusive predictive impact of hippocampal volumes on the DMN functional connectivity in a cluster located in the right hemisphere and covering part of the posterior temporal and inferior parietal lobes. This region is part of the anterior portion of the temporo-parietal junction (TPJ) territory[36]. The right TPJ (rTPJ) is an associative area involved in theory-of-mind processes[37,38], and in the conceptually similar construct of "mentalizing"[39]. As social-cognition skills, these abilities rely on "a network of areas at least partly overlapping with the DMN"[40], and the DMN itself is crucial for sustaining a skill like theory of mind[41]. It is, therefore, expected that reduced DMN strength in the rTPJ may lead to reduced abilities of social cognition in the AD trajectory. Unfortunately, no formal measure of social cognition was available for the participants included in this study. However, it is particularly significant that the strength of the DMN in the rTPJ was associated with performance on the recall part of the Prose Memory test with a $p < 0.01$. The short stories used to test verbal episodic learning (such as the logical-memory test of the Wechsler Memory Scale, or the Babcock story) are usually structured around events that are prone to elicit a strong empathic response (i.e., the robbery and parenting issues Anna Thompson has difficulties with, or the tragedy that strikes the victims of the flooding river). Similarly, theory of mind and TPJ function are tested via the administration of short stories that are conceptually equivalent[42]. Moreover, memory and theory-of-mind abilities share neuroanatomical correlates[43,44] and a tight bi-directional interplay exists between the two functions: more details about somebody's life story are remembered, more vivid is the use of theory-of-mind skills in that specific context[45]. Conversely, processing information via a social cognitive route improves encoding and memory performance[46]. Within this context, it is not a coincidence that recent evidence also shows that when encoding of information has a social connotation, it engages the rTPJ[47]. In summary, although social cognition abilities were not directly investigated in the present study, converging evidence indicates, beyond simple speculation, that a conceptual thread connects rTPJ, social cognition and memory, and that the *post hoc* association we found between memory performance and the strength of the DMN in the rTPJ may pave the way for the study of social cognition as a domain that could be discriminatory between normal ageing and AD. It is known that AD patients show a profound impairment in social cognition, which is independent of the impairment in general cognition[48]. Vice versa, healthy elderly adults show no decrement in theory-of-mind skills compared to young adults[49]. Moreover, difficulties in perspective taking contribute to increased anosognosia. A study of cortical metabolism found that the reduction of glucose uptake in the TPJ territory was associated with poorer

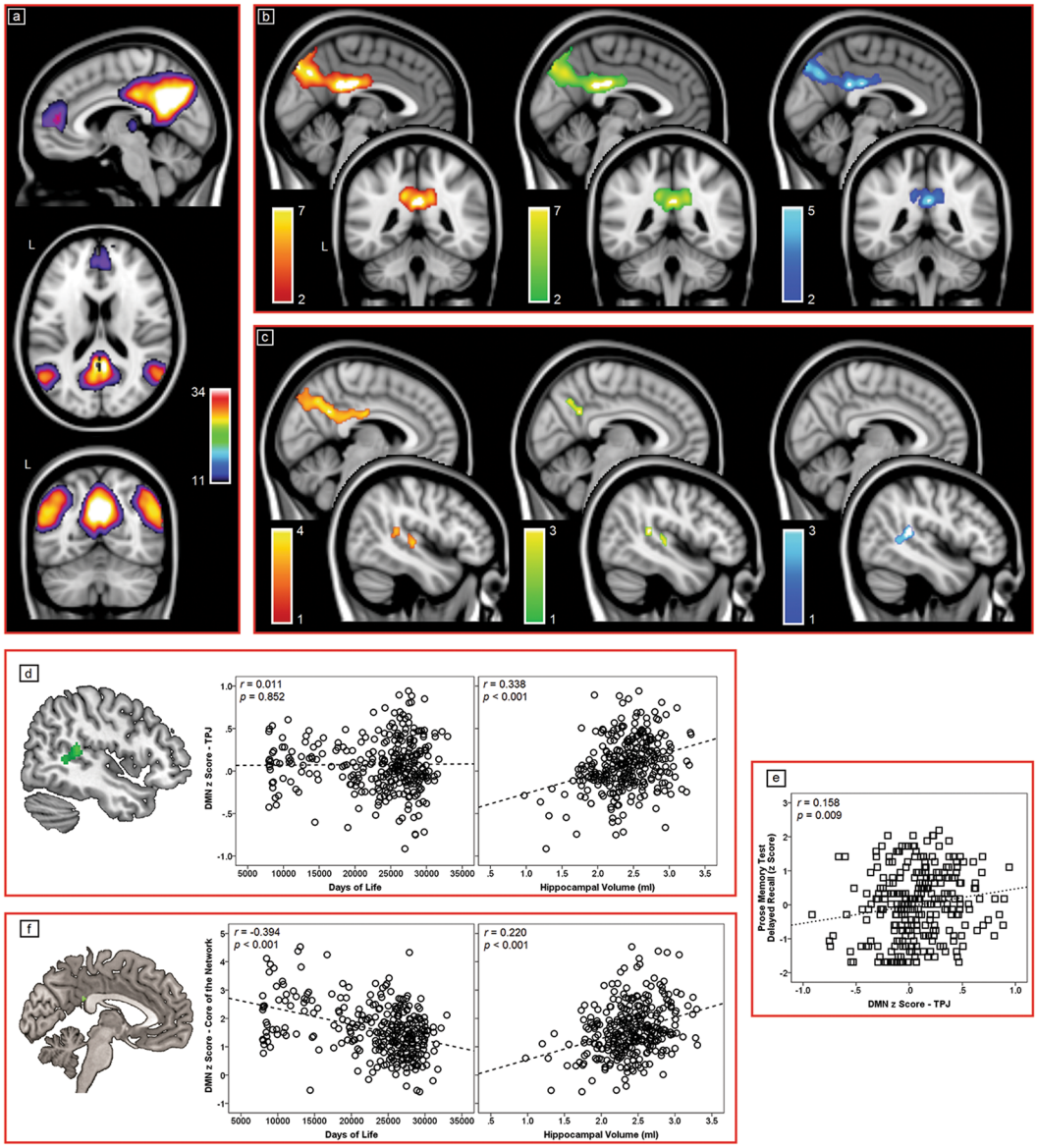

Figure 2. (**a**) The DMN map, as estimated with a one-sample *t* test carried out on the entire cohort and controlling for both proxies and all covariates (x = 6; z = 18; y = −62), and linear regression models testing the association between each of the proxies and functional connectivity of the DMN. Specifically, (**b**) the negative association between functional connectivity and the ageing proxy (x = −2; y = −44), and (**c**) the positive association between functional connectivity and the AD proxy (x = 6; x = 46). Uncorrected associations are shown in red, models corrected for the homologous proxy are shown in green, and the fully-corrected models are shown in cyan. *z* scores are indicated on the side of each output. (**d**) Association between functional connectivity of the DMN within the right TPJ (expressed as an average of *z* scores) and each of the two main independent variables of this study. (**e**) Association between the DMN signal in the right TPJ and performance on the Prose Memory test (delayed recall). Finally (**f**), the association between functional connectivity of each of the two proxies and the main DMN core. This core regions was located in the posterior cingulate cortex (BA 31, *p*FWE = 0.001, cluster extent: 12 contiguous voxels, peak Talairach coordinate: x = −4, y = −38, z = 26). The dotted lines represent linear associations. Pearson's *r* coefficients and respective *p* values are shown. DMN: Default-Mode Network; TPJ: Temporo-Parietal Junction.

disease awareness[50]. A solid link between anosognosia and DMN function exists. Autobiographical memory, envisioning of the future, and theory of mind are three examples of "DMN task" in which the personal perspective has to shift, and a "self-projection" is requested[1]. Along these lines, anosognosia can be considered, for all intents and purposes, a failure of self-projection. Based on this, loss of functional connectivity between the TPJ and computational hubs of the DMN will result into a disconnection between the ability to adopt a certain viewpoint during mentation and awareness of cognitive deficits, with an incidental effect on memory abilities when these are tested with tasks rich in empathy-inducing content.

Cluster Number	Cluster pFWE	Cluster size (voxels)	Z Score at Local Maximum	Side	BA	Region	Talairach Coordinates		
							x	y	z
Negative association between age and DMN									
1	0.000	2443	6.05	R	31	Cingulate Gyrus	2	−37	28
			6.00	R	7	Precuneus	2	−66	38
			5.45	L	23	Cingulate Gyrus	−2	−26	27
			4.97	L	7	Precuneus	−2	−75	44
			4.62	R	7	Precuneus	10	−75	50
			4.13	L	31	Precuneus	−12	−57	29
			4.10	L	31	Precuneus	−6	−49	30
			3.77	R	39	Middle Temporal Gyrus	30	−53	27
			3.76	L	31	Precuneus	−10	−45	34
2	0.043	351	3.74	R	13	Insula	42	−13	4
			3.63	R	41	Transverse Temporal Gyrus	53	−15	8
Positive association between hippocampal volume and DMN									
1	0.002	569	4.37	R	41	Transverse Temporal Gyrus	34	−36	15
			4.04	R	41	Superior Temporal Gyrus	50	−34	13
			3.87	R	41	Superior Temporal Gyrus	57	−29	11

Table 3. Distinctive and exclusive association between functional connectivity of the DMN and age/hippocampal volume. BA: Brodmann Area; L: Left; R: Right.

Although the TPJ is not usually considered a "prototypical" area distinctively affected by AD pathology (like the transenthorinal region, the hippocampus, or the posterior cingulate cortex), recent studies have described in explicit terms the TPJ as a central region significantly affected by AD pathological changes. Together with the posterior hippocampus and the posterior cingulate cortex, the "temporo-parietal junction seem to make an important contribution in the longitudinal progression during the very early stages of amyloid-β accumulation"[51, page2249]. Similarly, the TPJ is (together with the temporal pole) the region with the most elevated uptake of TAU binding tracer[52], and is characterised by reduced glucose metabolism both in early-onset and late-onset AD[53]. Altogether, these findings are highly convergent towards a link between alterations seen in the TPJ and the spectrum of pathological and neurofunctional alterations seen in AD.

This study is not free from limitations. On one hand, the choice of days of life at scan and hippocampal volumes as predictors offered important advantages. These include construct validity (i.e., the presence of a net theoretical association between each predictor and the construct the predictor is meant to quantify), excellent numerical variability and absence of floor or ceiling effects. However, other variables could be selected as indices more linked to physiological ageing or neurodegeneration (e.g., an index of cellular ageing such as telomere length, rather than "demographic ageing", and a measure of regional brain metabolism instead of brain morphology). Although we acknowledge our choices as theoretically sound and methodologically strong, we cannot rule out the possibility that other selections could have yielded small differences in the pattern of findings. Second, the idea of "independence" operationalised by our methodology was bound to to the concept of significant improvement in the model fit. No measurable variable exists that is exclusively associated with ageing (and not AD) or exclusively associated with AD (and not ageing). Statistics, however, offers a valuable method to study the prediction offered by a variable after partialling out the amount of variability predicted by a second variable of no interest and calculating thus its distinctive contribution to the model. Third, we identified the DMN as a single independent component based on haemodynamic regularities. Other studies separated the DMN into two or more subsystems[17,54,55], often labelled anterior DMN and posterior DMN. We analysed one pattern of connectivity only because we found only one map which showed the typical topography characteristics of the DMN.

Conclusion

In summary, although group-comparison models indicate that ageing and AD are associated with the statistical strength of activity in the DMN in a qualitatively similar manner, regression models provided statistical evidence indicating that the typical pattern of associations seen in postero-medial regions appears not to be a distinctive sign of neurodegeneration. Hippocampal volumes were instead predictive of DMN connectivity within the rTPJ. The role of this structure in social cognition and awareness of disease suggests the study of these symptoms deserves more attention for the development of a clinical marker able to detect sporadic AD in its early stage.

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Author contributions

Matteo De Marco: Contribution to study design, data analysis and drafting of manuscript. Sebastian Ourselin: Contribution to software for data analysis and critical review of manuscript. Annalena Venneri: Contribution to study design, critical review and finalising of manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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