Universidade do Minho
Escola de Medicina

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Neuroinflammation in early and late onset Alzheimer´s disease: a multimodal analysis

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Trabalho efetuado sob a orientação do
Professor Doutor Nuno Sousa
e do
Professor Doutor Manuel Melo Pires

novembro de 2017

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

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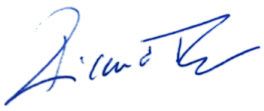
Signature: 

Aos meus pais.

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Abstract

Alzheimer´s disease (AD) is a chronic neurodegenerative disorder and is the most common cause of dementia. The two major neuropathological hallmarks of the disease are senile plaques, mainly composed of extracellular deposits of amyloid β (Aβ), and neurofibrillary tangles, consisting of intracellular aggregates of aberrantly phosphorylated tau protein. This is accompanied by synaptic loss and neuronal loss, dendritic and axonal changes, and inflammatory reactive lesions. Cumulative data suggests that neuroinflammation plays a prominent role in AD pathogenesis and age-associated dysregulation of the neuroimmune system. From a clinical point of view, despite the resemblance of neuropathological, there are important differences between the group of patients with sporadic early onset (<65 years old) and late onset Alzheimer's disease (>65 years old). Thus, it seems important to understand the age-dependent relationship between neuroinflammation and the underlying biology of AD in order to identify potential explanations for clinical heterogeneity, interpret biomarkers and to promote the best treatment for different clinical AD phenotypes.

In this thesis, it is first demonstrated that microglia activation has particular characteristics associated to AD that are distinct from frontotemporal dementia (FTD), another type of neurodegenerative dementia studied in this work. Subsequently, we found that the neuroinflammatory pathological markers (microglia activation and astrogliosis) in late stage AD human tissue have an overall similar pattern in both early and late onset AD, despite the greater severity of the pathological markers in the younger group. In a prospective clinical study, we showed a pro- and anti-inflammatory immune dysregulation in the cerebrospinal fluid cytokines in patients with AD and FTD, together with findings of particular signatures for each disorder. Furthermore, our results supports a possible protective role of inflammatory upregulation in early disease stages and suggest an age effect on IP-10 mediated pathogenesis in AD. The studies presented in this thesis call for a reappraisal of aging as a modulating factor in sporadic AD associated inflammation and highlight the idea that inflammation in this context is not exclusively detrimental or beneficial, but has to be fine-tuned. The study of this delicate balance in the different ages will be important to understand treatment efficacy, namely immunotherapeutic approaches, in clinical trials.

Resumo

A doença de Alzheimer (DA) é uma doença neurodegenerativa crônica e é a causa mais frequente de demência. As características neuropatológicas principais da doença são as placas senis, compostas principalmente por depósitos extracelulares de substância amilóide β (Aβ) e tranças neurofibrilhares, constituídas por agregados intracelulares de proteína tau fosforilada. Estes achados acompanham-se de perda neuronal e sináptica, alterações dendríticas e axonais e lesões inflamatórias reativas. A literatura suporta um papel proeminente da neuroinflamação na patogénese da DA, assim como a existência de uma desregulação do sistema neuroimune associada ao envelhecimento. Do ponto de vista clínico, apesar da semelhança dos achados neuropatológicos, existem diferenças importantes entre os doentes com DA esporádica de início precoce (<65 anos) e início tardio (> 65 anos). Assim, é importante compreender o papel da idade na relação entre neuroinflamação e a biologia subjacente da DA, para identificar possíveis explicações para a heterogeneidade clínica, interpretar os biomarcadores e promover o melhor tratamento para os seus diferentes fenótipos clínicos.

Nesta tese, demonstramos que a ativação da microglia possui características particulares associadas à DA, que foram distintas da demência frontotemporal (DFT), outro tipo de demência neurodegenerativa estudada neste trabalho. Posteriormente, verificamos que os marcadores patológicos de neuroinflamação (microglia ativada e astrogliose) apresentam um padrão similar na DA de início precoce e tardio, embora exiba maior severidade no grupo mais jovem. Num estudo clínico prospetivo, mostramos que existe uma desregulação imune anti- e pro-inflamatória pela análise de citocinas do líquido cefalorraquidiano em doentes com DA e DFT, juntamente com achados que sugerem uma assinatura específica para cada entidade. Além disso, os resultados suportam um possível papel protetor da hiper-regulação inflamatória nos estágios iniciais da doença e sugerem um efeito modelador da idade na patogénese da DA mediada pelo citocina IP-10. Os estudos apresentados nesta tese requerem uma reavaliação do envelhecimento como modulador na inflamação associada à DA esporádica e fortalecem que essa inflamação não é exclusivamente prejudicial ou benéfica, mas tem que ser ajustada. O estudo deste equilíbrio nas diferentes idades será importante para entender a eficácia de tratamentos, nomeadamente nos baseados em imunoterapia, em ensaios clínicos.

CONTENTS

ABBREVIATIONS LIST

Aβ - Amyloid beta

ABRA – Amyloid-β related angiitis

AD - Alzheimer´s disease

ApoE – Apolipoprotein E

APP – Amyloid precursor protein

CAA - Cerebral amyloid angiopathy

CD33 – Sialic acid-binding immunoglobulin lectin 3

CSF – Cerebral spinal fluid

DRS-2 - Dementia rating scale-2

EOAD - Early onset Alzheimer´s disease

FDG - Fluorodeoxyglucose

FGF - Fibroblast growth factor

FTD – Frontotemporal dementia

G-CSF - Granulocyte colony-stimulating factor

GM-CSF - Granulocyte-macrophage colony-stimulating factor

IL- Interleukin

INF - Interferon

IP-10 - Interferon γ induced chemokine 10

LOAD - Late onset Alzheimer´s disease

MCP-1 - Monocyte chemotactic protein 1

MMSE - Mini mental state examination

MIP - Macrophage inflammatory protein

MRI – Magnetic resonance imaging

PCA – Posterior cortical atrophy

PDGF - Platelet-derived growth factor

PET - Positron-emission tomography

PSEN1 - Presenilin 1

PSEN2 - Presenilin 2

TNF - Tumor necrosis factor

TREM 2 - Triggering receptor on myeloid cells 2

TSPO - Translocator protein-18 kDa

VEGF - Vascular endothelial growth factor

1. Introduction

In this chapter, the concept of neuroinflammation in aging and Alzheimer´s disease (AD) is reviewed, with particular emphasis on the differences between early and late onset AD, and the interplay between brain aging, neuroinflammation and AD phenotypes. Part of this revision has been published (Taipa et al., 2016)

1.1. General

Alzheimer´s disease is a chronic neurodegenerative disorder and is the most common cause of dementia worldwide, accounting for 50-70% of cases (Winblad et al., 2016). Because the primary risk factor for AD is old age, the prevalence of the disease is increasing dramatically with ageing populations worldwide and represents significant health-care cost in developed countries (Reitz et al., 2011; Winblad et al., 2016). An estimated 40 million people, the majority older than 60 years, have dementia worldwide, and this figure is projected to double every 20 years, until at least 2050.

The classical clinical presentation of AD includes a gradual and progressive decline of cognitive domains, with prominent memory impairment and executive dysfunction interfering with daily life activities. Atypical presentations of AD include language, visual, praxis, or executive problems before, and more pronounced, than memory deficits (McKhann et al., 2011; Scheltens et al., 2016). The former is the typical presentation in elderly patients and the later more common in early onset AD.

The two major neuropathological hallmarks of the disease are senile plaques, which are mainly composed of extracellular deposits of amyloid β (Aβ) and neurofibrillary tangles, which consist of intracellular aggregates of aberrantly phosphorylated tau protein. This is accompanied by neuronal and synaptic loss, dendritic and axonal changes, and inflammatory reactive lesions (Cummings and Cummings, 2004; Taipa et al., 2012).

Despite the significant advances that have been made in the understanding of AD pathogenesis, it remains largely unknown. After two decades of the amyloid cascade hypotheses proposed by Hardy and Higgins (Hardy and Higgins, 1992), multiple lines of research still support Aβ aggregation as the critical step that initiates AD pathology. The strongest evidence for Aβ aggregation as a causative factor comes from studies of

familial Alzheimer's disease cases with mutations of amyloid precursor protein (APP), presenilin 1 (PSEN1) or presenilin 2 (PSEN2).

Cumulative data suggests that neuroinflammation plays a prominent and early role in AD (Heneka et al., 2015). The amyloid cascade-inflammatory hypothesis proposes that Aβ induces an inflammatory response that is enhanced by the presence of tau (Bolós et al., 2017). The inflammatory response is driven principally by activated microglia, the predominant resident immune cell in the central nervous system (CNS) (Norden and Godbout, 2013; Ransohoff, 2016).

More recently, studies have highlighted the biological process of age related changes associated to microglial cells (Flanary et al., 2007; Mosher and Wyss-Coray, 2014; Streit et al., 2009) and suggested that microglial senescence can be directly associated to neurofibrillary degeneration (Streit et al., 2014). Despite clinical resemblance and similar neuropathological findings, there are important differences between the group of patients with sporadic early onset (<65 years old) and late onset Alzheimer's disease (>65 years old). Thus, it seems important to understand the age-dependent relationship between neuroinflammation and the underlying biology of AD in order to identify potential explanations for clinical heterogeneity, interpret biomarkers and to promote the best treatment for different clinical AD phenotypes.

1.2. Neuroinflammation in aging and Alzheimer´s disease

1.2.1. Brain immune system

Microglia are the resident immune cells of the CNS and considered the tissue-resident macrophages. Nissl, who first described these cells in 1899, distinguished microglia from other neural cells based on the shape and their nuclei (Nissl, 1989). Microglia arise from myeloid precursors and constitute an autonomous population distinct from the peripheral circulating mononuclear phagocytes (Ginhoux et al., 2010). These cells account for up to 16% of total cell CNS population and this is dependent on the brain region (Norden and Godbout, 2013). There is limited replication and turnover of microglia, suggesting that microglia are a very long-lived and stable cell population (Mosher and Wyss-Coray, 2014; Norden and Godbout, 2013). Microglia can offer several macrophage related activities that provide an innate immune response as the first and

main form of active immune defense in the brain (Norden and Godbout, 2013). The term microglial activation encompasses the process where microglia change shape, molecular signature, and cellular physiology in order to respond to injury or disease (Kettenmann et al., 2011). Resting microglia are characterized by a small cell body, highly ramified processes with weak expression of associated cell surface marker antigens (Derecki et al., 2014). In contrast, activated microglia display shortened processes and hypertrophy of cell body (Perry et al., 2010). The definition of resting microglia does not mean a passive spectator in the healthy adult CNS. In vivo two-photon microscopy imaging studies, showed that microglia survey the brain parenchyma by constantly extending and retracting their processes, and react rapidly to brain injury or insult, and are more properly termed “surveillant” (Davalos et al., 2005; Malm et al., 2015; Nimmerjahn et al., 2005). The functions of microglia in the normal healthy brain beyond immune surveillance are unclear, but recently functions that are more sophisticated have been described such, as participating actively in the maintenance and plasticity of neuronal circuits, contributing to the protection and the remodeling of synapses (Ji et al., 2013; Lourbopoulos et al., 2015).

Microglial activation states have been classically described as activated (M1) or alternatively activated (M2) (Martinez et al., 2008). The M1 phenotype is characterized by production of proinflammatory cytokines, such as IL-1β, TNF-α and IFN-γ, whereas in the M2 phenotype microglia secrete anti-inflammatory cytokines, such as IL-4, IL-10 and transforming growth factor-β, which downregulate inflammation and promote tissue remodeling/repair and angiogenesis (Czeh et al., 2011). However, this categorizing system relies on peripheral macrophages studies, which do not recapitulate all microglial functions. Microglial cells show high levels of diversity and plasticity and their classification into an M1 and M2 polarized state is likely an oversimplification (Malm et al., 2015; Ransohoff, 2016). Recently, it has been proposed that microglia switch continuously between phenotypes (Heneka et al., 2015; Town et al., 2005). Another type of neuroimmune cells are the perivascular macrophages (Hickman and El Khoury, 2013). They seem to be derived from circulating macrophages, and are able to perform all the known functions of peripheral macrophages; they undergo complete turnover approximately every 3 months (Audoy-Rémus et al., 2008; Bechmann et al., 2001). Circulating blood monocytes can enter the CNS, but it is not clear how often it happens

under non-inflammatory conditions. In conditions of disrupted blood brain barrier, and when properly stimulated, they can differentiate into microglia-like cells or perivascular macrophages morphologically and phenotypically (Hickman and El Khoury, 2013).

Astrocytes are the most abundant glial cells in the central nervous system and their function is critical for the support of neuronal homeostasis (Sofroniew and Vinters, 2010). The term astrogliosis describes a wide range of both molecular and functional changes in astrocytes aimed to neuroprotection and repair of injured neural tissue (Osborn et al., 2016; Sofroniew and Vinters, 2010) . It has been shown that that reactive astrogliosis and glial scar formation play essential roles in regulating CNS inflammation (Sofroniew and Vinters, 2010). Reactive astrocytes in response to different kinds of insult can produce molecules with either pro- or anti-inflammatory potential. Additionally, reactive astrocytes can exert both pro- and anti-inflammatory effects on microglia (Burda and Sofroniew, 2014; Farina et al., 2007). More recently, in an analogy to the M1/M2 macrophage nomenclature, two different types of reactive astrocytes, named A1 and A2, were described (Liddelow et al., 2017). A1 astrocytes upregulate many classical complement cascade genes previously shown to be destructive to synapses and A2 upregulate neurotropic factors. Interestingly, this group showed that A1 astrocytes are induced by activated microglia (Liddelow et al., 2017).

In summary, microglia and astrocytes are fundamental players of the brain immune system and their (dys)functions are highly interdependent.

1.2.2. Neuroinflammation in the brain aging

Brain aging is a dynamic process that adapts to different external and internal challenges (Lövdén et al., 2013). There is clinical and experimental evidence that neuroinflammation in the aged brain is characterized by a shift towards a pro-inflammatory state (Barrientos et al., 2010; Norden and Godbout, 2013). Additionally, aging is associated to an imprecisely defined process of “immunosenescence” that affects both adaptive and innate immune systems (Di Benedetto et al., 2017).

Inflammation in the brain is defined by upregulated astrocyte and microglial cell reactivity in association with increased levels of circulating cytokines such as TNFα, IL-1β and IFN-γ (McGeer and McGeer, 2001; Ojo et al., 2015; Streit et al., 2004). With aging,

microglia phenotype shifts progressively towards the activated form, together with enhanced sensitivity to inflammatory stimuli (priming phenomena) (Norden and Godbout, 2013; Perry and Teeling, 2013). In normal human brain aging, microglia is characterized by up-regulation of glial activation markers such as IL-α (Sheng et al., 1998) and major histocompatibility complex II (MHC II) (Sobel and Ames, 1988). MHC II is important because it is conserved across species and its presence is interpreted as indicative of microglial priming (Norden and Godbout, 2013). There is compelling evidence from different research groups and aging models, that following different types of challenge (bacteria, virus, stress, surgical intervention), aged animals exhibited a clear and exaggerated neuroinflammatory response, when compared to young adult animals (Abraham et al., 2008; Barrientos et al., 2010; Godbout et al., 2005; Rosczyk et al., 2008). These studies provided evidence that over the lifespan, episodes of systemic inflammation and cytokine stimulation can "instruct" microglia and increase their reactivity (Barrientos et al., 2010; Lourbopoulos et al., 2015). Interestingly, some of these sensitized neuroinflammatory responses are specific to the hippocampal formation, which is important for memory function (Barrientos et al., 2010). Microglia from the aged CNS could be described as hyper-vigilant to disturbances in central homeostasis with less capability of shifting among functional states. Proteins expressed in CNS microenvironment, which are known to inhibit microglia activation or pro-inflammatory immune responses, have been implicated in the mechanism of how microglia becomes chronically sensitized during normal aging (Biber et al., 2007). In fact, various proteins have been described that activate anti-inflammatory signals following ligand receptor interactions (Griffiths et al., 2009), particularly CD200 (Barrientos et al., 2015; Hoek et al., 2000; Lyons et al., 2007) and fractalkine (CX3CL1) (Barrientos et al., 2015; Corona et al., 2010; Wynne et al., 2010); interestingly, both are preferentially expressed in neurons. These proteins inhibit microglia through their cognate receptor, which is expressed predominantly in myelomonocytic cell types. During aging, the expression of levels of these ligands decreases concurrently with increases in microglial activation status. More recently, another line of research suggests that significant and prolonged elevation in hippocampal corticosterone (the endogenous glucocorticoid in rodents) leads to microglial priming (Barrientos et al., 2015). However, the simplistic view that aging CNS shifts microglial polarization from alternative M2 state to the

classical, proinflammatory state, should be interpreted cautiously because many studies found that both M1 markers and M2 markers are increased in aged mice (Mosher and Wyss-Coray, 2014). For example, active microglia from aged mice actually had higher levels of IL-10 production (an anti-inflammatory cytokine) than those of adult mice and lower expression of TGFβ (an inflammatory cytokine) (Sierra et al., 2007). In this case, the maintenance of inflammatory response could be attributed to an impaired response to IL-10 in the aged brain (Norden and Godbout, 2013). Furthermore, primed microglia phenomena have been described mainly in mouse models (Norden and Godbout, 2013; Raj et al., 2014), and less in human brain research (Sheng et al., 1997). More recently, studies have shown that the cerebrospinal fluid (CSF) levels of YKL-40 (a microglial marker) increases in normal aging (Alcolea et al., 2015; Olsson et al., 2013; Sutphen et al., 2015).

Together with this perspective that microglia becomes primed and more reactive with age, others have shown that microglia become senescent and less reactive with age (Flanary et al., 2007; Streit et al., 2014, 2009). In the heathy young CNS, microglia have a typical ramified morphology and are distributed throughout the neural parenchyma in a "space-filling" manner (Wong, 2013). Due to the prolonged lifespan of CNS microglia they are more susceptible to accumulate aging-related changes (Gehrmann and Banati, 1995), such as in their distribution, morphology and behavior (Mosher and Wyss-Coray, 2014; Wong, 2013) (table 1). Many microglial cells in the aged brain show dystrophic features indicative of age-related alterations. These dystrophic microglia have de-ramification or decreased arborization of their processes, loss of finely branched cytoplasmic processes, cytoplasmic beading/spheroid formation, shortened and twisted cytoplasmic processes and in some instances there is partial or complete cytoplasmic fragmentation (Streit et al., 2004). More recently, shortening of microglial cell processes and reduced of coverage of brain parenchyma with aging has been reported, however, without microglia dystrophy or changes in cell density. (Davies et al., 2016). The meaning of these morphological changes or why they happen is still to be understood.

There is limited knowledge about ageing of astroglia and the data available is controversial. In human post-mortem tissue analysis there was no significant changes in astroglial cell counts between old and young adult brains (Fabricius et al., 2013; Pelvig et al., 2008). In old rodents, an increase, a decrease and no change in the number of

GFAP positive astroglial cells have been reported (Verkhratsky et al., 2016). Some data supports that aged astrocytes show characteristics of the senescence-associated secretory phenotype, which involves increased secretion of inflammatory components (Salminen et al., 2011).

In summary, aged microglia are primed with exaggerated and prolonged responses to inflammatory stimuli and display dysfunctional dystrophic age associated features. Yet, it is still to be determined if microglia activation is the cause of neurodegeneration or a secondary reactive (beneficial) process; or if the neurodegeneration is actually secondary to microglia senescence and associated loss of microglial protection.

Table 1

Summary of principal changes associated with microglial aging, adapted from Wyss-Coray (Wyss-Coray et al., 2006) and Wong (Wong, 2013).

Changes in microglial distribution
Replicative senescence (reduced mitotic activity in response to CNS injuries)
Decreases in regularity in distribution
Changes in morphology
Decrease in individual microglial ramification (dendritic arbor area, branching, and total process length)
Appearance of morphological changes suggestive of increased activation state (shortened and extensively branched processes and hypertrophy of cell body)
Appearance of dystrophic microglia (de-ramified, fragmented, or tortuous processes, cytoplasmic beading/spheroid formation)
Changes in microglial dynamic behavior and function
Decrease in the motility and migration process
Changes in intercellular signaling and marker expression (MHC II, CD11b)
Impaired phagocytosis
Impaired proteostasis

1.2.2. Neuroinflammation in Alzheimer´s disease

After two decades of the amyloid cascade hypotheses proposed by Hardy and Higgins (Hardy and Higgins, 1992), multiple lines of research still support the Aβ aggregation as the critical step that initiates AD pathology. However, despite being required, it appears that Aβ aggregation alone is not sufficient for the development of the neuropathological and clinical syndrome of AD (Musiek and Holtzman, 2015). Several research studies report links between AD and genes regulating immunity as well as the expression of immune factors in blood, CSF and brain tissue (Eikelenboom et al., 2010; Heneka et al., 2015; International Genomics of Alzheimer's Disease Consortium (IGAP), 2015; Mhatre et al., 2015; Xiang et al., 2006). There is data supporting that neuroinflammation in Alzheimer's disease is not a passive mechanism activated by senile plaques and neurofibrillary tangles, but instead contributes, as much or even more, to pathogenesis as do plaques and tangles (Heneka et al., 2015; Mhatre et al., 2015; Zhang et al., 2013). Epidemiological studies indicate that systemic markers of the innate immunity are risk factors of late-onset AD (Dik et al., 2005; Schmidt et al., 2002; Wichmann et al., 2014; Yaffe et al., 2003). More recently, inflammation in AD gained strong support from genome-wide association studies that identified genes involved in inflammation that are associated with increased risk of developing AD (Schellenberg and Montine, 2012), including *TREM2* (triggering receptor on myeloid cells 2) (Guerreiro et al., 2013; Korvatska et al., 2015) and *CD33* (sialic acid-binding immunoglobulin lectin 3) (Bradshaw et al., 2013; Griciuc et al., 2013). Prospective cohort studies have suggested that elevations in inflammatory mediators may be present years before clinical disease onset (Engelhart et al., 2004; Schmidt et al., 2002; Tan et al., 2007). However, other longitudinal studies reported no associations between inflammation and AD risk (Ravaglia et al., 2007; Sundelöf et al., 2009). Furthermore, non-steroidal anti-inflammatory drug (NSAID) epidemiology and clinical trials showed mostly negative results, playing against the importance of inflammation in AD pathogenesis (Jaturapatporn et al., 2012). However, these disappointing results are not surprising if one takes into account that normal physiological cytokine regulation of glial activation and microglial phenotypes are highly dependent on the context and the disease stage (Heneka et al., 2015).

The activated immune response associated with AD, that leads do the chronic production of inflammatory cytokines close to the AD associated pathology in brain tissue, can be detected in CSF and peripheral blood. The levels of cytokines, their receptors and other proteins associated with immune responses in blood and CSF of AD patients have been frequently investigated (Brosseron et al., 2014) and the cytokines IL-1β, IL-6, and TNF-α seem to increase slightly but steadily over the time during the course of AD (Brosseron et al., 2014).

More recently, studies have consistently found an increase in CSF YKL-40 levels in AD. They also found a correlation between CSF YKL-40 levels with markers of neurodegeneration, such as tau, and with at-risk ε4 carriers during mid middle age (Alcolea et al., 2015; Olsson et al., 2013; Sutphen et al., 2015). Lipocalin-2 (LCN2), a member of the lipocalin protein family, became increasingly relevant in recent years as a biomarker in several diseases, including Alzheimer´s disease (Choi et al., 2011). In the brain, LCN2 expression was found to occur mainly in response to an injury or inflammatory status (Marques et al., 2008). Regarding the inflammatory response in the context of AD associated pathology, it was recently showed that LCN2 production was up-regulated in both choroid plexus epithelial cells and astrocytes (Mesquita et al., 2014). Additionally, the same study showed that Aβ toxicity for astrocytes required the presence of LCN2 (Mesquita et al., 2014). Furthermore, it was shown that AD patients present altered levels of LCN2 in the CSF (Naude et al., 2012). LCN2 production by reactive astrocytes appears to be a critical step for the course of the disease, whether by potentiating or attenuating the inflammatory response (Ferreira et al., 2015).

A recent meta-analysis found evidence to suggest elevated peripheral levels of IL-1β, IL-2, IL-6, IL-18, sTNF-R1, sTNF-R2, homocysteine, hsCRP, IFN-γ, CXCL-10, EGF, VCAM-1, α1-antichymotrypsin and transferrin and decreased levels of IL-1Ra transferrin and leptin in patients with AD compared with healthy controls, highlighting the role of peripheral inflammation in AD pathology (Lai et al., 2017). The authors also emphasize that there was significant heterogeneity in most comparisons (Lai et al., 2017). Actually, the data obtained from different studies (either in CSF or blood) is controversial, particularly due to the different methodological approaches and to the lack of longitudinal studies (Brosseron et al., 2014).

Neuropathological studies have shown the presence of a broad variety of inflammation-related proteins (complement factors, acute-phase proteins, proinflammatory cytokines) and clusters of activated microglia around amyloid plaques (Figure 1) in AD subjects and also in AD mice models (Eikelenboom et al., 2010), and these findings have been implicated in the process of neurodegeneration (Akiyama et al., 2000; Meda et al., 1995).

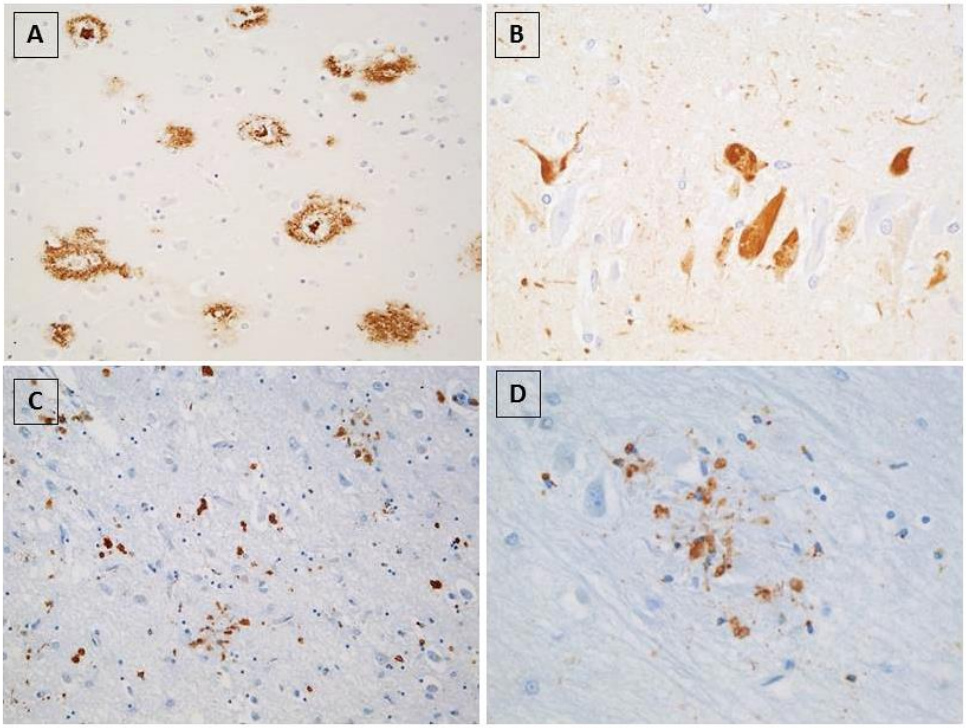


Fig 1. Alzheimer´s disease neuropathology. A - Senile plaques and globose diffuse deposits demonstrated with anti-Aβ antibody (M 0804, Dako). B – Neurofibrillary tangles demonstrated by phosphorylated tau protein immunohistochemistry (PHF-Tau; AT8, Thermo Scientific); C – Diffuse distribution of activated microglia in the cortex with clustering within and around amyloid plaques; D – Higher magnification of amyloid plaque with activated microglial in the CA4 region of the hippocampus (C and D: CD68 immunohistochemistry; PGM1 clone, Dako).

Neuropathological studies also showed that the neuroinflammatory response in the neocortex is present in the early stages of AD pathology and precedes the late stage,

tau-related pathology (Webster et al., 2006). Furthermore, microglial activation has been shown to progress with the clinical stage of dementia, with neuropathological stage of disease severity, and with stage of progression of Aβ plaques (Mrak, 2012; Vehmas et al., 2003; Xiang et al., 2006). In vivo imaging studies, using ^{11}-C-*R*-PK1195 PET ligand, showed that activated microglia accumulate near the amyloid plaque pathology(Edison et al., 2008). Correlation analysis between these ligands and AD severity provided heterogeneous findings, with studies showing that activated microglia burden correlates with cognitive decline (Edison et al., 2008; Kreisl et al., 2016) while other study found an opposite correlation (Hamelin et al., 2016).

The pathological accumulation of Aβ is considered the key factor that drives neuroinflammation responses in Alzheimer´s disease (Heneka et al., 2015). The chronic deposition of amyloid-β stimulates the persistent activation of microglial cells in Alzheimer's disease (Prinz et al., 2011). Microglia undergoes a progressive switch from a neuroprotective M2 status to a classically activated phenotype M1, characterized by production of proinflammatory cytokines (Wang et al., 2015). The persistent microglia activation and consequently microglia-derived cytokine overexpression, caused by continuous formation of Aβ and positive feedback loops between inflammation and amyloid precursor protein processing, can increase Aβ production and decrease Aβ clearance, ultimately causing neuronal damage (Heneka et al., 2015; Mrak, 2012; Wang et al., 2015). In addition, ongoing exposure to Aβ, chemokines, cytokines and other inflammatory mediators can be responsible for the functional impairment of microglial cells seen at plaque sites (Krabbe et al., 2013; Streit et al., 2009) and thus impede the protective role of microglia in Aβ clearance (Hickman et al., 2008). Recently, Kim et al. (Kim et al., 2013), showed that soluble Aβ oligomers impair synaptic plasticity and cause synaptic loss in mouse AD models and brains of AD patients binding to the murine PirB (paired immunoglobulin-like receptor B) and its human ortholog LilrB2 (leucocyte immunoglobulin-like receptor B2) receptors, respectively. The PirB receptor was first described exclusively in the immune system but is now know to be expressed by neurons.

Microglia can have different roles and effects depending on the particular disease stage and which brain region is affected in each model (Heneka et al., 2015). Alzheimer´s

disease mouse models studies showed that in younger ages, together with the appearance of the first Aβ plaques, the microglia is activated towards the alternative state and at older ages, together with the increased accumulation of extracellular oligomeric Aβ, there is a widespread microglial activation toward the classic phenotype (Jimenez et al., 2008). Recently, Sudduth et al. described in the early-stage AD brains an apparent polarization toward either M1 or M2 brain inflammatory states (Sudduth et al., 2013). The M2 polarized group had greater numbers of neuritic plaques, eventually reflecting disease progression. The heterogeneity found in the early stage AD can influence the response to therapeutic agents that act on immune system and inflammation (Sudduth et al., 2013). The neuropathological study of AD patients that had undergone active amyloid-β vaccination as part of the AN1792 trial showed significantly reduced levels of β-amyloid, reduction of aggregated tau in neural processes (not in neurofibrillary tangles) and, although there was no difference in the total microglial load, there were reduced levels of a range of activated microglial species when compared to patients who died from AD without treatment (Boche et al., 2010; Zotova et al., 2013). These findings suggest that downregulation of microglial activation through amyloid-β immunotherapy possibly reduces the inflammatory component of the neurodegeneration of Alzheimer's disease (Boche et al., 2010). However, a different line of research supports that aging-related microglial degeneration and loss of microglial neuroprotection rather than microglial activation contributes to the onset of sporadic Alzheimer´s disease (Streit et al., 2009). Furthermore, a recent in vivo imaging study using a second-generation 18-kDa translocator protein positron emission tomography radiotracer, showed that microglial activation appears at the prodromal and possibly at the preclinical stage of AD, and seems to play a protective role in the clinical progression of the disease at these early stages (Hamelin et al., 2016). A role for peripheral-derived macrophage cells in AD pathophysiology have recently coming into attention (El Khoury et al., 2007). There is extensive evidence that blood-derived monocytes can phagocytose Aβ (Hohsfield and Humpel, 2015) and that these cells can be recruited to the AD brain, albeit in low numbers (Lampron et al., 2013).

Reactive astrocytes tend to accumulate around fibrillary amyloid plaques (Medeiros and LaFerla, 2013). Similar to microglia, astrocytes release cytokines and other potentially cytotoxic molecules after exposure to Aβ thus aggravating the neuroinflammatory

response (Heneka et al., 2015). Glial cell activation can be an early event in the Alzheimer´s disease process, even preceding Aβ deposition. Recently, Rodriguez-Vieitez and colleagues (Rodriguez-Vieitez et al., 2016), using a PET tracer for astrocytes (^{11}C-deuterium-L-deprenyl), showed prominent initially high and then declining astrocytosis in autosomal dominant Alzheimer's disease carriers, contrasting with the increasing amyloid-β plaque load during disease progression. This study provided *in vivo* evidence that astrocyte activation is a very early feature of, at least familial, Alzheimer's disease pathology (Rodriguez-Vieitez et al., 2016). Other lines of research have linked senescent astrocytes to the increased risk of sporadic AD (Bhat et al., 2012). In transgenic AD animals studies, astrocyte atrophy was reported in both hippocampus (Olabarria et al., 2010) and entorhinal cortex (Yeh et al., 2011). Interestingly, while astroglial atrophy appears as a generalized process, the astrocytes that surround plaques were hypertrophic with increased surface and volume of GFAP-immunostained profiles (Olabarria et al., 2010).

In summary, the role of microglia remains controversial in AD pathogenesis and the question of whether activated microglia aid in promoting clearance of toxic Aβ species or if their proinflammatory profile exacerbates pathology is currently a topic of debate (Schott and Revesz, 2013). Although there is broad evidence of a large immune response component in AD, it remains to be completely solved the issue of which activation phenotype affects the onset or progression of the disease and, consequently, which should be the therapeutic target (Varnum and Ikezu, 2012). In addition, the questions regarding the role of excessive astrogliosis or astrocyte senescent loss of function in AD pathogenesis remain to be solved (Medeiros and LaFerla, 2013).

1.3. Early and late onset Alzheimer´s disease

Regardless of the similar neuropathological features, important differences exist between early and late onset AD (EOAD and LOAD) patients (Koedam et al., 2010; Möller et al., 2013; Rabinovici et al., 2010). The separation of EOAD from LOAD at 65 years old is a conventional cutoff point indicative of a sociological partition in terms of employment and retirement, but there is no specific biological significance in the use of

this specific age, and there is a range of disease features that do not respect this arbitrary division (Koedam et al., 2010; Rossor et al., 2010). However, this arbitrary cutoff point has been used widely by different research groups and allowed the uniform study of AD patients with different ages of onset.

1.3.1. Clinical presentation

Clinical diagnosis of any dementia syndrome depends on taking a history from the patient and their caregivers, neuropsychological testing, and assessment of symptoms with time (Scheltens et al., 2016). Memory impairment is the most common syndromic presentation of AD dementia (McKhann et al., 2011). Atypical forms of AD include the less frequent but well defined clinical phenotypic variants of non-amnestic focal cortical syndromes (language, visuospatial, executive) (Dubois et al., 2014; McKhann et al., 2011; Scheltens et al., 2016).

Whether age of onset defines the clinical presentation of AD has been a matter of debate for decades and reports on this issue are often contradictory. Nonetheless, some differences have been consistently recognized. Earlier onset is associated with a worse prognosis and a faster progression (Lam et al., 2013). Younger-onset patients have comparatively worse outcomes in the MMSE at baseline, show a steeper cognitive and functional decline and seem to have higher mortality risks when compared to older-onset patients (Jacobs et al., 1994; Koedam et al., 2008; Panegyres and Chen, 2013). In addition, different patterns of cognitive deficits are apparent; non-amnestic presentations are more often found in early onset disease, described in 33-64% of EOAD compared to 6-12.5% of LOAD patients (Koedam et al., 2010; Mendez et al., 2012).

Earlier neuropsychological studies have shown that younger patients have more language disability when compared to older-onset patients (Chui et al., 1985; Filley et al., 1986; Seltzer and Sherwin, 1983). The risk of having language difficulties detected by caregivers has also been shown to nearly duplicate for each 10-year decrease in AD patients' age (Koss et al., 1996). Other groups have recognized a greater impairment in measures of attention, praxis and visuo-construction tasks in EOAD (Frisoni et al., 2005; Fujimori et al., 1998; Suribhatla et al., 2004). On the other hand, LOAD patients seem to consistently have preferential memory involvement (Gour et al., 2014; Kaiser et al.,

2012; Möller et al., 2013). More recently, a research group showed that EOAD and LOAD groups showed distinct patterns of memory impairment (Joubert et al., 2016). Despite both groups being similarly affected on measures of episodic, short term and working memory, semantic memory was significantly more impaired in LOAD than in EOAD patients.

To explore the relation between this clinical duality and pathologic features, Murray et al (Murray et al., 2011) divided a cohort of AD patients into "hippocampal sparing", "limbic predominant" and "typical AD" according to neurofibrillary pathology distribution. They have shown that a younger age of onset (mean 63 years) was associated with greater neurofibrillary tangle burden in cortical association areas and that older age (mean 76 years) was more often associated with limbic predominant pathology. The hippocampal sparing group had greater prevalence of atypical presentations and a faster cognitive decline, similar to what has been described in EOAD. Seizures and extrapyramidal features seem to be more frequent in EOAD (Amatniek et al., 2006; Chui et al., 1985). There are contradictory reports of other symptoms in both groups. For example, there are reports of higher anxiety levels in EOAD (Porter et al., 2003), while others have shown greater neuropsychiatric and behavioural symptoms in LOAD, including anxiety, depression, agitation, hallucinations and delusions (Toyota et al., 2007; van Vliet et al., 2012).

Limited research has been reported into sex differences in brain aging, particularly of the neuroinflammation process. However, gender effect is an interesting issue due to the differences of the neuroendocrine milieu and its possible relation to inflammation cascades (particularly steroid related pathways). The dynamic change in hormonal status in women during the menopause transition may promote a dysregulation of cellular processes involved in hypothalamic-pituitary-adrenal axis and thus have potential implications on stress mediated neurotoxicity (Bale and Epperson, 2015). It is also important to recognize the importance of immunological differences in males and females within the CNS at different development time points and their possible relevance for the susceptibility in the development of neurological conditions later in life (Hanamsagar and Bilbo, 2016). A recent study in mice by Mangold and colleagues showed a greater induction of MHC class I components and receptors with aging, this finding being greater in females than in males (Mangold et al., 2017). However, despite

the prevalence of AD being greater in women, the prevailing view has been that this difference is due to the fact that women live longer than men on average, and older age is the greatest risk factor for Alzheimer's. Many studies of incidence of Alzheimer's have found no significant difference between men and women in the proportion who develop Alzheimer's at any given age (Alzheimer's Association, 2015).

1.3.2. Biomarkers

Biomarkers are parameters (physiological, biochemical, anatomic) that can be measured in vivo and that reflect specific features of disease-related pathophysiological processes (Jack et al., 2011). AD biomarkers can be related specifically to the presence of amyloid (CSF Aβ42 or PET amyloid) and tau pathology (CSF or PET tau), or reflect "downstream" damage by selective topographical brain involvement, either by brain atrophy measure by magnetic resonance imaging (MRI) structural analysis or hypometabolism of neocortical regions measured by fluorodeoxyglucose (FDG)—PET (Dubois et al., 2014). MRI studies show that younger-onset patients have greater generalized neocortical atrophy than LOAD subjects when compared to healthy controls (Frisoni et al., 2007; Gour et al., 2014). This is in accordance with glucose metabolism studies, which demonstrate a premature decline in glucose metabolism and a more severe and widespread hypometabolism in EOAD (Kim et al., 2005). Regarding regional differences, older patients tend to have a more circumscribed involvement, with preferential reduction in the hippocampus and related structures, including the amygdala (Cavedo et al., 2014), retrosplenial and temporoparietal junction volumes (Frisoni et al., 2007), while younger patients tend to have a greater temporoparietal and parietooccipital grey matter atrophy (Frisoni et al., 2005; Möller et al., 2013). White matter atrophy mimics this pattern (Canu et al., 2012). Moreover, both perfusion and glucose metabolism studies have shown a predilection for temporo-parietal-occipital association areas in early AD versus medial temporal cortex susceptibility in late AD (Kaiser et al., 2012; Kemp et al., 2003). Interestingly, another study has shown no significant difference in total or regional amyloid burden, measured by Pittsburg compound-B PET, despite showing decreased glucose metabolism in bilateral temporoparietal and occipital cortex in EOAD. This finding suggests that both early amyloid-β and increased susceptibility to

pathology in younger onset patients might be responsible for cortical dysfunction in EOAD (Rabinovici et al., 2010). The greater involvement of hippocampal related structures in LOAD is also apparent in functional connectivity studies that have shown that older patients have decreased activation of the anteromedial temporal network, correlating with poorer performance in memory tasks; EOAD was associated with less activation of the dorsolateral prefrontal network, manifested by worse performance on executive function tasks (Gour et al., 2014).

CSF pathophysiological markers for AD include decreased levels of Aβ1–42 and increased levels of total tau and hyperphosphorylated tau. The combined use of these biomarkers is associated with significant sensitivity and specificity in the diagnosis of AD (Dubois et al., 2014). There is some evidence that EOAD patients have a greater reduction of Aβ1-42 (and corresponding greater elevation of tau) than LOAD patients when compared to young and old controls, respectively, although no differences emerge in the direct comparison between EOAD and LOAD (Bouwman et al., 2009). Others have reported lower levels of Aβ1-42 in EOAD (Andreasen et al., 1999) or no differences (Möller et al., 2013; Ossenkoppele et al., 2015b). A study comparing CSF biomarkers in different EOAD subtypes, including amnestic, logopenic progressive aphasia and posterior cortical atrophy (PCA) found no differences in the Aβ levels, but showed that PCA was associated with lower levels of total tau and phosphorylated tau (Teng et al., 2014).

1.3.3. Genetics

Amyloid precursor protein, presenilin 1 and presenilin 2 mutations can cause autosomal dominant AD, and although they may be present in up 71% of familial cases, they account for only about 1-5% of all AD patients. These patients typically have an early or very early onset disease (<45years) (Campion et al., 1999; Scheltens et al., 2016; van der Flier et al., 2011). Apolipoprotein E (ApoE) ε4 is the major genetic risk factor for AD. For ApoE ε4 homozygotes lifetime risk for AD is more than 50% and for ApoE ε3ε4 carriers is around 20-30%, compared with 11% for men and 14% for women overall irrespective of ApoE genotype (Genin et al., 2011). It is usually associated with greater hippocampal atrophy and a poorer performance in memory based tasks (Murray et al., 2011; van der

Flier et al., 2011) and it decreases the age of onset by up to 2.45 years for each copy of the allele (Naj et al., 2014; van der Flier et al., 2011). Conversely, non-ApoE ε4 patients tend to have greater structural and clinical involvement of non-hippocampal, neocortical areas (Murray et al., 2011). ApoE ε4 allele carriers among AD patients are most frequently found in the 60-69-year-old range (Davidson et al., 2006), therefore including both older EOAD patients and younger LOAD patients. The ApoE ε2 allele is less frequently found in AD patients than in normal controls and there seems to be no difference in its prevalence between EOAD and LOAD (Davidson et al., 2006). Genome wide association studies have identified several other risk genes for LOAD. The association between nine of them (PICALM, CLU, CR1, BIN1, CD2AP, EPHA1, MS4A4A, CD33 and ABCA7) has been shown to account for 1.1% of age of onset variation, versus 3.9% of variation provided by ApoE. The most significant association was found for the CR1, BIN1 and PICALM genes (Naj et al., 2014). Another candidate gene that may have an impact on age of onset is DCHS2, a gene expressed in the cerebral cortex (Kamboh et al., 2012). Yet, and surprisingly, these genetic variants do not seem to bring significant value for the distinction between EOAD and LOAD, as they simply seem to anticipate pathology.

1.4 Interplay between brain aging, neuroinflammation and AD phenotypes

AD prevalence is strongly associated with increasing age and aging changes in microglia have been hypothesized to play a prominent role in disease pathogenesis (Wong, 2013). Recently, the consistent pattern of increases in YKL-40 level with aging supports the concept that neuroinflammation is a process that occurs normally with aging (Alcolea et al., 2015; Olsson et al., 2013; Sutphen et al., 2015). The additional finding of a stronger association with at-risk ε4 carriers during mid middle age suggests that this age-related process may be further exacerbated in the presence of insults including amyloid deposition and neuronal injury (Sutphen et al., 2015). There are important clinical differences between sporadic EOAD and LOAD. Taking into account the data regarding the importance of neuroinflammation in the pathogenesis of AD, and the differences of the neuroimmunological milieu of the aged brain, it is conceivable that the neuroinflammation associated with AD can, at least in the beginning, differ between

these two groups and contribute to the clinical differences. Not many studies have addressed this issue.

Hoozemans et al (Hoozemans et al., 2011) compared the presence of microglia and astrocytes, in clinically and pathologically confirmed AD and non-demented control cases in relation to age. In their study they suggested that the association between neuroinflammation and AD is much stronger in relatively young patients as compared to the older patients (age at death <80 vs >80 years old). Kreisl et al (2016) reported higher ^{11}C-PBR28 (an 18kDa translocater protein of second generation, used as microglia marker) binding in EOAD patients than LOAD patients, both at baseline and follow-up (Kreisl et al., 2016). Microglial activation increases with the neuropathological stage and disease severity (Vehmas et al., 2003; Xiang et al., 2006). A key issue would be to know if inflammation differs between these two groups (EOAD vs LOAD) at different pathological and clinical AD stages.

Another remarkable finding is that, in contrast to AD, activated microglia are not found in the similar-appearing Aβ diffuse deposits of the brains of neurologically normal elderly individuals (Mackenzie et al., 1995). One of the possibilities is that for those unusual elderly individuals with only diffuse Aβ deposits there is an inherent difference in the responsiveness of microglia (Mrak, 2012). Curiously, plaque-associated microglia were not seen in diffuse plaque-only young Down´s syndrome brain (Stoltzner et al., 2000). This subgroup of cases was from very young patients (between 12 and 29 years old), possibly supporting the notion that Aβ inflammatory response may also differ in the very young. More recently, a study showed that in Down´s syndrome patients with AD pathology (>40 years old) there is a distinct neuroinflammatory phenotype compared to sporadic Alzheimer's disease due to microglia bias toward an M2b phenotype (Wilcock et al., 2015). Interestingly, a recent study using an ex vivo model by co-culturing organotypic brain slices from an AD mouse model (APPPS1) and young, neonatal wild-type (WT) mice, showed that exposing old microglial cells to conditioned media of young microglia or addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) was sufficient to induce microglial proliferation and reduce amyloidplaque size (Daria et al., 2017). Clinicopathological studies from brain donation programs showed that the presence of moderate and severe Alzheimer´s-disease type pathology changes is more associated to dementia in younger old persons than in older

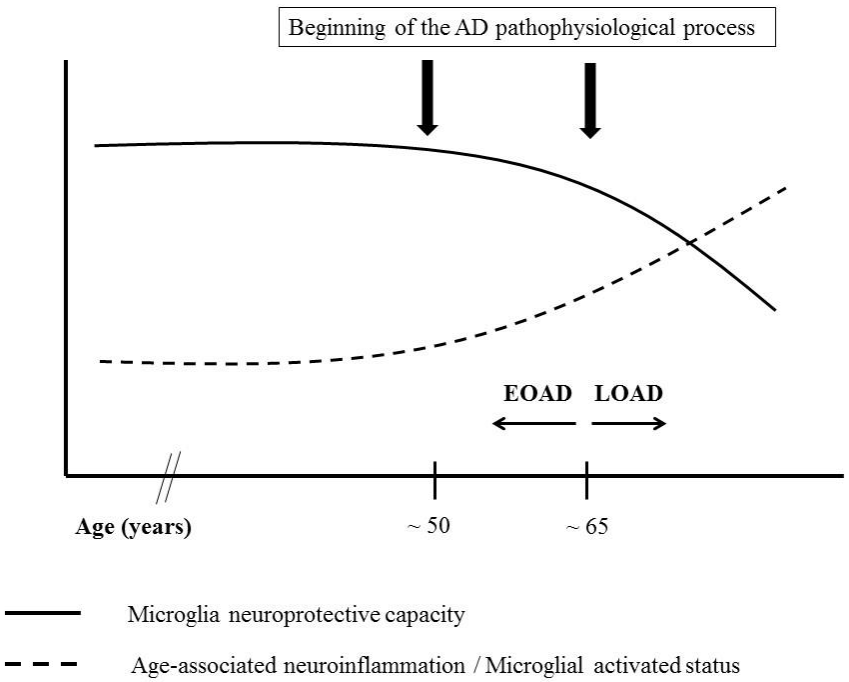
old persons (Savva et al., 2009). These findings suggest that additional factors are involved in the clinical expression of dementia in the oldest old, such as variable tolerance to neuropathological lesions (Savva et al., 2009). We speculate that a different neuroinflammation apparatus in this age can partial explain this discrepancy.

The study of inflammatory cytokines in CSF as biomarkers of AD has shown very different and contradictory results between different research groups (Wang et al., 2015). The analysis of different neuroinflammation-related proteins in the blood, including several interleukines (IL-1α, Il-1β, IL-6, IL-10), α2-macroglobulin, brain-derived neurotrophic factor (BDNF), complement factor H and heat shock protein 90 (Hsp90) has not shown significant differences between EOAD and LOAD, but studies are scarce and with small sample numbers (Dursun et al., 2015; Gezen-Ak et al., 2013). Tumor necrosis factor alpha (TNFα) levels have been shown to be both higher and lower in EOAD (Alvarez et al., 1996; Gezen-Ak et al., 2013).

Some of the risk loci in modifying age of disease onset identified in genome wide association studies have recognized roles in the immune system, including phagocytosis and immune cell trafficking (Rosenthal and Kamboh, 2014). Both CLU and CR1 encode for proteins that regulate complement activation; EPHA1, mostly expressed in leukocytes, is involved in T cell regulation; ABCA7 is highly expressed in the hippocampal neurons and in microglia and is involved in Aβ processing; and CD33, overexpressed in AD patient's microglia, encodes for an endocytic receptor that takes part in cell-cell interactions and in immune cell regulation (Nuutinen et al., 2009; Rosenthal and Kamboh, 2014). TREM2, another identified loci associated with increased risk for AD, is involved in immune response (Guerreiro et al., 2013). There are studies that found a significantly earlier onset of symptoms in patients with TREM2 variants (Slattery et al., 2014), but others found only an association to shortened disease duration and not to age of onset (Korvatska et al., 2015). Aβ cerebral amyloid angiopathy (CAA) and particularly Aβ related angiitis (ABRA), is other AD related clinical feature that bridges AD, inflammation and age. CAA describes a group of biochemically and genetically diverse disorders, which have in common the deposition of amyloid in media and adventitia of cortical and leptomeningeal vessels (Revesz et al., 2009). Sporadic CAA and AD have overlapping biology with shared risk factors (Yamada, 2002). Aβ vascular deposition affects about 30% of the otherwise normal elderly and over 90% of those

with Alzheimer's disease, in whom CAA tends also to be more severe (Revesz et al., 2009; Scolding et al., 2005). ABRA is characterized by a vasculitic transmural, often granulomatous, inflammatory infiltrates affecting leptomeningeal and cortical vessels that have abundant amyloid-beta deposition within the vessel walls (Salvarani et al., 2013; Scolding et al., 2005). The recent finding of autoantibodies against Aβ1-40 and 1-42 forms of amyloid in the CSF of 2 patients with ABRA and inflammation associated to CAA (DiFrancesco et al., 2011; Hermann et al., 2011), together with the description of meningoencephalitis caused by active or passive immunotherapeutic approaches to reduce Aβ burden in AD (Orgogozo et al., 2003), suggests that an immune response directed against Aβ may represent a common disease mechanism shared by ABRA and in complications of therapy for AD (Salvarani et al., 2013). The mean age of presentation of ABRA is lower than that of sporadic non-inflammatory Aβ-related CAA (66 vs 76 years, respectively) (Salvarani et al., 2013; Scolding et al., 2005). These findings support a role for the interactions between age, and inflammation in AD related pathophysiology and clinical expression.

In summary, the pathophysiological mechanisms underlying the clinical differences between EOAD and LOAD are still not well known, but the differences of neuroinflammation characteristics with aging may help to partially explain it (Figure 2).



2. Aims of the PhD Thesis

This Thesis project aimed to investigate the interplay between the immune system and the disease progression of patients with early and late onset sporadic AD. The key question to be answered was to know if neuroinflammation associated to AD is different in early and late onset patients. Additionally, to address the issue of the possibility of a disease specific signature of neuroinflammation in AD, another neurodegenerative dementia (FTD) was studied for comparison. The tasks of the Thesis were:

a) Pathological study

- Analysis of the microglia activation pattern in different regions of AD and FTLD pathologically confirmed cases.
- Analysis of pathological neuroinflammatory markers (microglia and astrocytes) in clinically and pathologically confirmed AD patients with different ages of onset.

b) Clinical study

- Analysis of the inflammatory profile of AD and FTD cases.
- Correlate the inflammatory profile with clinical phenotype, neuropsychological and clinical progression within the AD group according to the age of onset.

3. Experimental work

3.1. Pathological study

For this task pathological proven AD, FTLD and non-demented aged matched controls cases were obtained from different Brain Banks (Portuguese Brain Bank, Manchester Brain Bank, Oxford Brain Bank and Queen Square Brain Bank). The table below summarize the details of the cases used for this task.

Table 2. Pathological study. Clinical and demographic characteristics.

Cases	Brain Bank	Pathology diagnosis	Gender	Braak stage	PMD	Age Onset	Age Death
1	Manchester	AD (EO)	M	VI	na	58	67
2	Manchester	AD (EO)	M	V-VI	na	58	68
3	Manchester	AD (EO)	M	VI	na	58	70
4	Manchester	AD (EO)	M	V-VI	na	54	59
5	Manchester	AD (EO)	M	V	na	53	66
6	Manchester	AD (EO)	M	VI	96	59	69
7	Manchester	AD (EO)	M	VI	na	55	65
8	Manchester	AD (EO)	F	VI	130	54	61
9	Manchester	AD (EO)	M	VI	na	na	71
10	Manchester	AD (EO)	M	VI	125	61	73
11	Manchester	AD (EO)	M	V-VI	96	57	66
12	Manchester	AD (EO)	M	VI	79	37	45
13	Manchester	AD (EO)	M	V-VI	61,75	52	65
14	Manchester	AD (EO)	M	V-VI	131	63	70
15	Manchester	AD (EO)	M	V-VI	75	55	64
16	Manchester	AD (EO)	F	VI	64	64	71
17	Manchester	AD (EO)	M	VI	81	59	72
18	Manchester	AD (EO)	M	VI	107	64	73
19	Manchester	AD (EO)	M	V-VI	36	60	73
20	Manchester	AD (LO)	F	V-VI	72	82	89
21	Manchester	AD (LO)	M	V-VI	na	71	76
22	Manchester	AD (LO)	M	V	96	75	83
23	Manchester	AD (LO)	F	VI	72	80	88
24	Manchester	AD (LO)	F	VI	176	77	86
25	Manchester	AD (LO)	F	V	72	79	88
26	Manchester	AD (LO)	F	V-VI	60	80	87
27	Manchester	AD (LO)	M	VI	96	75	82
28	Manchester	AD (LO)	F	VI	52	67	76
29	Manchester	AD (LO)	M	VI	96	NA	76
30	Manchester	AD (LO)	F	V	25,5	74	81
31	Manchester	FTLD-TDP A	M	na	na	49	58
32	Manchester	FTLD-TDP A	M	na	na	60	68
33	Manchester	FTLD-TDP A	M	na	na	59	64
34	Manchester	FTLD-TDP A	M	na	na	69	75
35	Manchester	FTLD-TDP A	M	na	na	54	66
36	Manchester	FTLD-TDP A	M	na	na	64	72
37	Manchester	FTLD-TDP A	F	na	45,5	66	72
38	Manchester	FTLD-TDP A	M	na	96	78	82
39	Manchester	FTLD-TDP A	M	na	94,5	63	65
40	Manchester	FTLD-TDP A	M	na	81	54	65
41	Porto	FTLD-TDP B	M	0-I	na	60	63
42	Porto	FTLD-TDP A	M	0	< 24	54	61
43	Porto	FTLD-TDP C	F	I	6	65	78

Cases	Brain Bank	Pathology diagnosis	Gender	Braak stage	PMD	Age Onset	Age Death
44	Manchester	CONT y	F	0	92,5	-	53
45	Oxford	CONT y	M	I-II	48	-	69
46	Oxford	CONT y	F	I-II	48	-	60
47	Oxford	CONT y	F	I-II	48	-	69
48	Oxford	CONT y	M	I	24	-	63
49	Oxford	CONT y	F	I	51	-	71
50	Oxford	CONT y	F	0	60	-	62
51	Oxford	CONT y	M	I	33	-	51
52	Oxford	CONT y	M	0	48	-	68
53	Oxford	CONT y	M	I	38	-	56
54	Oxford	CONT y	M	I-II	24	-	59
55	QSBB	CONT y	F	0-I	82	-	56
56	QSBB	CONT y	M	0	80,35	-	38
57	QSBB	CONT y	F	0	79	-	64
58	QSBB	CONT y	F	I-II	29,5	-	53
59	Manchester	CONT o	F	IV	na	-	81
60	Manchester	CONT o	M	IV	48	-	92
61	Manchester	CONT o	F	I-II	72	-	88
62	Manchester	CONT o	M	0-I	24	-	92
63	Manchester	CONT o	M	I-II	12	-	85
64	Manchester	CONT o	F	0	41,5	-	90
65	Manchester	CONT o	M	I-II	12	-	95
66	Oxford	CONT o	M	III	23	-	77
67	Oxford	CONT o	F	0-I	21	-	80
68	Oxford	CONT o	F	I-II	29	-	79
69	Oxford	CONT o	F	0	48	-	81

3.1.1. Analysis of the microglial activation pattern in different regions of AD and FTLD pathologically confirmed cases.

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Patterns of Microglial Cell Activation in Alzheimer Disease and Frontotemporal Lobar Degeneration

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Keywords
Alzheimer disease · Frontotemporal lobar degeneration · Microglia · Hippocampus · Memory

Abstract
Aims: Microglia-driven neuroinflammation can play an important role in the pathophysiology of neurodegenerative disorders. In this study, we sought to characterize the distribution of microglial cell activation in 2 neurodegenerative dementias with distinct protein signatures, Alzheimer disease (AD) and frontotemporal lobar degeneration (FTLD) of the TDP subtype, and to determine if there was an anatomical correlation with the phenotypes most commonly associated with these conditions. ***Methods:*** The distribution and extent of microglial cell activation was assessed semiquantitatively in the hippocampal formation, cortical gray matter, and subcortical white matter of CD68-immunostained sections of the frontal, temporal, parietal, and occipital cortices from 15 pathologically confirmed cases of AD, 13 cases of FTLD, and 18 controls. ***Results:*** Significantly higher levels of microglial cell activation occurred in the subiculum in AD and FTLD than in controls. Additionally, AD had higher microglial activation in the CA1 and FTLD in the hippocampal white matter than the controls. Microglial activation was greater in the dentate gyrus molecular layer in AD than in FTLD. In the cortical regions, the 2 pathological groups differed only in frontal white matter, with the FTLD group showing higher microglial scores. FTLD showed higher microglial activation in the white matter compared to the respective gray matter in the entorhinal, temporal, and frontal regions. ***Conclusions:*** Our work expands the knowledge of the distribution and magnitude of microglial activation in these disorders. Additionally, we found some microglial circuit-specific patterns that could help to explain some of the clinical overlap between AD and FTLD-TDP, namely in memory deficits.

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Introduction

Microglial cells are the predominant resident immune cells of the central nervous system and are considered as tissue-resident macrophages. The functions of microglia

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in the normal healthy brain beyond immune surveillance are unclear, but, recently, more sophisticated functions such as participating actively in the protection and remodeling of synapses were described [1, 2]. In addition, microglial activation occurs early in the pathogenesis of neurodegenerative diseases [3, 4].

Alzheimer disease (AD) is the most common cause of dementia worldwide, followed by frontotemporal lobar degeneration (FTLD) in people younger than 65 years of age [5]. Both AD and FTLD are characterized by the accumulation and aggregation of misfolded proteins. Whereas AD pathology is characterized by extracellular amyloid beta (Aβ) plaques and intracellular aggregates of aberrantly phosphorylated tau protein, i.e., neurofibrillary tangles (NFT), the pathology of FTLD is more heterogeneous. Approximately half of the cases are defined by the presence of tau-immunoreactive changes (NFT-like structures, Pick bodies, or glial inclusions) [6], and have been designated as FTLD-tau [7]. Most of the remaining cases of FTLD are associated with the presence of various combinations of immunoreactive TDP43 neuronal cytoplasmatic inclusions, dystrophic neurites, and neuronal intranuclear inclusions [6], and such cases have been designated as FTLD-TDP [7]. It is believed that the abnormal protein accumulation can trigger a brain inflammatory reaction, inducing the production of a series of proinflammatory mediators and microglial activation [8, 9].

Cumulative data suggest that neuroinflammation plays a prominent and early role in AD [8]. Neuropathological studies have shown the presence of a broad variety of inflammation-related proteins (complement factors, acute-phase proteins, and proinflammatory cytokines) and clusters of activated microglia around amyloid plaques in AD subjects [10]. In vivo imaging studies, using the 11-C-R-PK1195 PET ligand, showed that activated microglia accumulate near the amyloid plaque pathology, and that activated microglia burden correlates with cognitive decline in AD [11]. Using the same ligand, in a small cohort of FTLD patients, activated microglia were detected in the typically affected frontotemporal brain regions [12]. Recently, Lant et al. [13], in a post-mortem immunohistochemistry (IHC) study, showed higher levels of microglial activation in the frontal and temporal cortices, and the white matter of FTLD patients.

Previous neuropathological studies were focused on individual neurodegenerative disorders and limited brain regions, and so they did not address possible regional differences in microglial activation within the central nervous system or pathologies. Taking into account the microglia-driven neuroinflammation role in the pathophysiology of neurodegenerative disorders, we sought to extend previous studies, regarding the characterization of the distribution of microglial cell activation in these 2 neurodegenerative dementias. We selected the TDP43 pathological subtype of FTLD to compare 2 distinct "proteinopathies." TDP43 pathology was found to be present in AD [14] and it has been shown to be associated with typical AD characteristics such as episodic memory loss and hippocampal atrophy [15]. Furthermore, the FTLD-TDP subtype has been associated with more severe episodic memory, and hippocampal atrophy may be a potential biomarker for FTLD-TDP in vivo [16]. AD cases with TDP43 pathology were excluded from the analysis.

Patient and Methods

Patients

Forty-six cases were investigated. The majority were obtained from the Manchester Brain Bank (15 AD, 10 FTLD, and 4 controls) through appropriate consent procedures for the collection and use of human brain tissues. Additionally, 14 control cases were obtained from the Oxford Brain Bank and 3 FTLD cases were obtained from the Portuguese Brain Bank, also through appropriate consent procedures for the collection and use of human brain tissues. Cases were approximately age-matched. The AD cases met the pathological criteria for definite AD ("high" AD neuropathologic change) [17]. Cases with associated hippocampal sclerosis and/or TDP43 pathology were excluded. The FTLD cases met the pathological criteria for FTLD [18] and all cases were FTLD-TDP (11 FTLD-TDP type A, 1 type B, and 1 type C) [7]. The 18 controls were judged to be clinically normal and none showed any pathology beyond that which might be anticipated for their age.

Methods

Immunohistochemistry

The IHC staining for CD68 was performed using the Ventana OptiView DAB IHC detection kit and BenchMark Ultra processor (Ventana, Tucson, AZ, USA). Sections of the temporal (including hippocampus and parahippocampal region), frontal, parietal, and occipital cortices were cut at 6-μm thickness from formalin-fixed, paraffin-embedded blocks and mounted on to glass slides. Paraffin tissue sections were deparaffinized with EZ Prep (Ventana) at 75°C for 16 min and pretreated with heat treatment with Ultra Cell Conditioning Solution (CC1, Ventana), and then the endogenous peroxidase was inactivated before incubation with the CD68 antibody (PG-M1, 1:400, Dako, Glostrup, Denmark) for 24 min at 36°C. Subsequently, the slides were incubated with OptiView HQ linker for 8 min, and with OptiView universal HRP multimer (Ventana) for 8 min at 36°C. Tissue sections were incubated with OptiView universal DAB chromogen (Ventana) for 8 min to detect the antigen-antibody complex and then counterstained with hematoxylin II (Ventana).

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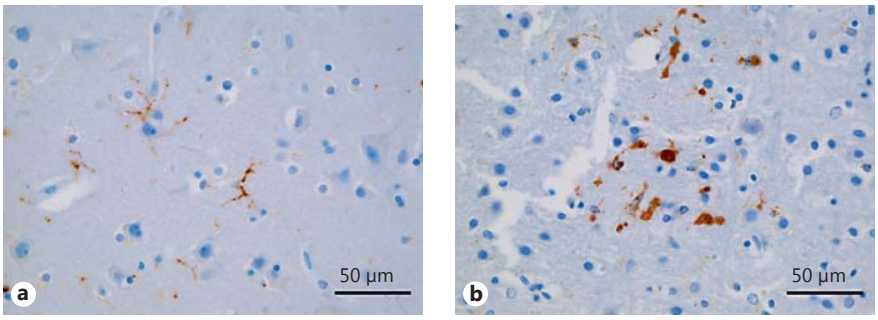

Fig. 1. Ramified (**a**) and activated (**b**) microglia cells. CD68.

Similar study methods for IHC were used with Iba1 antibody (Wako cat. No. 019-19741) in a random series of frontal sections from across the groups (15 cases).

Microscopic Analysis

The CD68 and Iba1 antibodies mark microglial cells, both in a resting and activated condition (Fig. 1). Sections of the different regions of interest were assessed for the presence of immunostained microglial cells within both the cortical gray matter and the subcortical white matter at ×20 magnification. As previously described [13], the frequency and "severity" (in terms of morphological types, with activated microglial cells being considered to be more severe than ramified microglial cells) of CD68-immunostained sections were assessed according to the following criteria: 0 = no immunostained cells present; 1 = very few immunostained cells present, all as ramified microglia; 2 = a moderate number of immunostained cells present, mostly ramified but some activated cells present; 3 = many, diffusely spread, immunostained cells present, all as activated microglia; 4 = many, large clusters of activated microglial cells present (Fig. 2). The perivascular CD68-immunostained cells (perivascular macrophages) were not considered for the score analysis.

In each section, scores were given for the cortex and the white matter. Additionally, in the temporal block, scores were given for hippocampal formation (CA1, CA2/3, the dentate gyrus [DG] granular cell layer, the DG molecular layer [DG-ML], and the subiculum), the hippocampal white matter, the entorhinal cortex, and the entorhinal white matter.

The assessment of Iba1 scores followed the same methods as for CD68.

All assessments were made by a single observer (R.T.), who was blinded to diagnosis. Sections were scored twice to increase objectivity, and discrepancies were reconciled by consultation with a second observer (M.M.P.). In addition, a random set of 8 sections was scored on a weekly basis over the course of the study.

Statistical Analysis

Rating data were entered into an Excel spreadsheet and analyzed using SPSS software v22.0. A p value <0.05 was considered statistically significant. The Kruskal-Wallis test was performed for analysis. If this detected any significant differences between the groups, post hoc testing was performed to identify the groups between which significant differences existed (by pairwise comparison). The Friedman two-way analysis and the Wilcoxon signed-rank test were used to compare different areas within each group.

Results

Three groups were established (Table 1) based on confirmation from pathology; cases were grouped as AD (n = 15), FTLD (n = 13) and nondemented controls (n = 18). There were no differences between AD, FTLD, and controls in age at death. The AD and FTLD groups do not differ in age at onset or disease duration ($p > 0.05$) (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000457127).

Histological Analysis

Resting microglia are characterized by a small cell body and ramified processes with a weak expression of associated cell surface marker antigens [19]. This "ramified" microglial cell phenotype is seen when no apparent tissue pathology is present (Fig. 1a). In response to injury or disease, microglia change into a macrophage-like phenotype (Fig. 1b), displaying shortened processes and hypertrophy of the cell body [3].

As previously described [13], the topographic distribution of activated microglial cells generally followed that of the principal pathological changes within the cortex and hippocampal formation. In the AD group, activated microglial cells were often clustered within and around amyloid plaques. The number of morphologically activated microglial cells was also high in the subcortical white matter, with a more dispersed distribution. In the FTLD group, activated microglial cells were more evident in the upper (I–III) than in the lower (IV–VI) cortical layers, and more so in the subcortical white matter than in the cortical gray matter (particularly in the entorhinal, temporal, and frontal areas).

In the controls, activated microglial cells were largely absent in most of the cases, though a few were occasionally seen in association with rare amyloid deposits. Ramified microglial cells were commonly seen in many of these cases.

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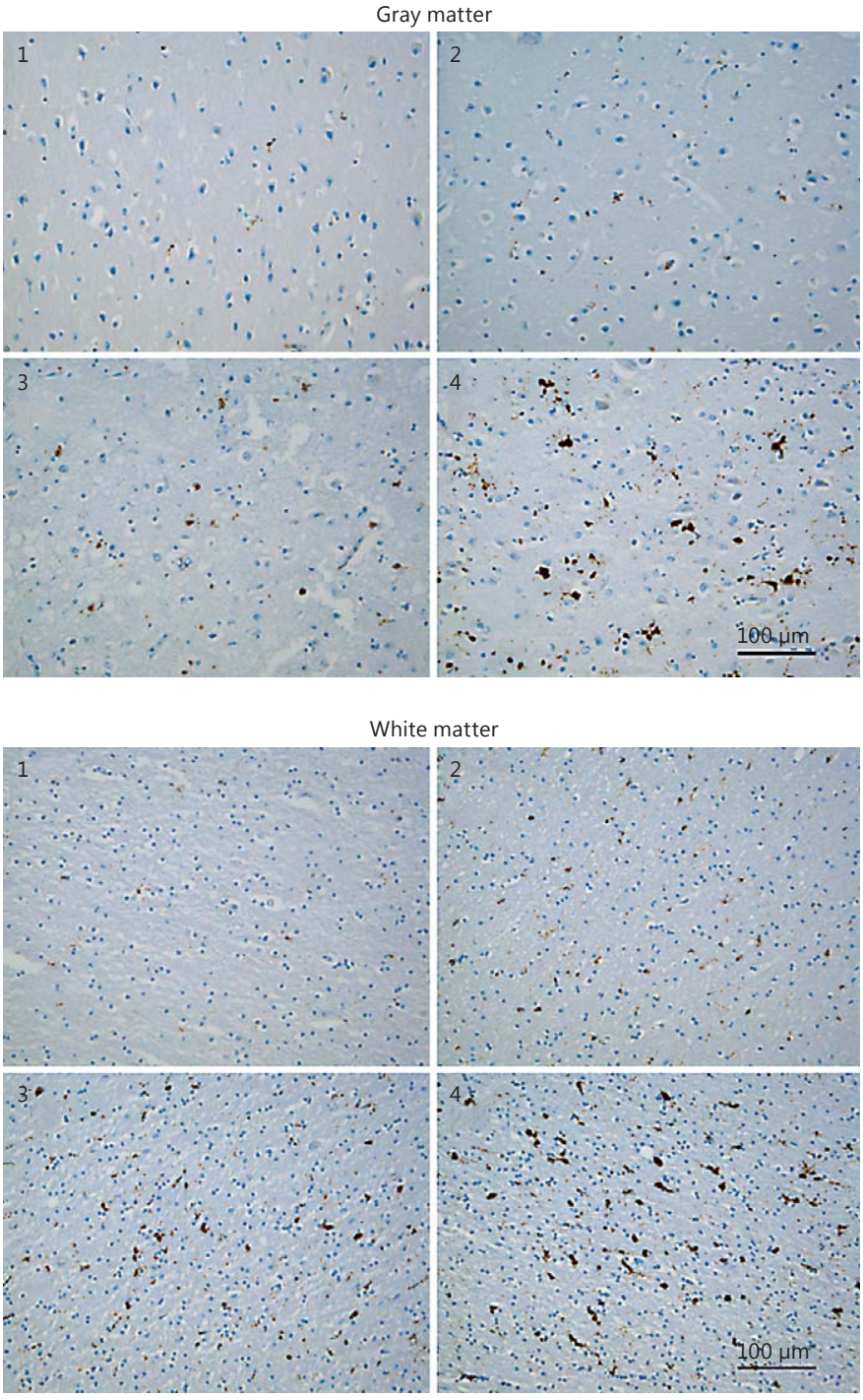

Fig. 2. Representative examples of the microglial assessment according to the scale description. CD68.

The morphological patterns were similar with both antibodies, with Iba1 showing a better definition of the ramified morphology of the resting microglia (online suppl. Fig. 1). In the sections analyzed with both antibodies, microglial scores showed a good correlation between them ($r = 0.609$, $p < 0.01$ in frontal cortex; $r = 0.514$, $p < 0.05$ in frontal subcortical white matter, the Kendall τ coefficient; online suppl. Fig. 2). The CD68 antibody was used as the principal microglial marker in the study for better comparison with the original study of the group [13].

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Microglial Scores Based on the Patterns of CD68 Staining

Hippocampal Formation

Microglial cell scores were significantly different across the AD, FTLD, and control groups for the CA1 region of the hippocampus ($p = 0.037$), the DG-ML ($p = 0.016$), the subiculum ($p = 0.002$), and the hippocampal white matter ($p = 0.016$). Pairwise comparisons showed that the AD group had higher microglial scores than the control group in CA1 ($p = 0.031$) and subiculum ($p = 0.005$). The FTLD group had higher microglial scores than the control group in the subiculum ($p = 0.022$) and hippocampal white matter ($p = 0.014$), and, interestingly, lower microglial scores in the DG-ML ($p = 0.043$). The scores of the 2 pathological groups (AD and FTLD) differed only in the DG-ML, with the AD group showing higher microglial scores ($p = 0.026$) (Fig. 3).

Cortex and Subcortical White Matter

Microglial cell scores were significantly different in the AD, FTLD, and control groups for the entorhinal cortex and white matter ($p = 0.036$ for both), the temporal cortex ($p = 0.007$), and the frontal cortex and white matter ($p = 0.047$ and $p = 0.007$, respectively). There was no significant effect of disease status in the other regions studied (temporal white matter, parietal cortex and white matter, occipital cortex and white matter).

Pairwise comparisons showed that the AD group had higher microglial scores than the control group in the entorhinal cortex ($p = 0.030$) and temporal cortex ($p = 0.007$). The entorhinal white matter showed a trend ($p = 0.053$) of higher values in the AD cases. The FTLD group had higher microglial scores than the control group in the frontal white matter ($p = 0.012$) and a trend in the frontal cortex ($p = 0.053$). The 2 pathological groups (AD and FTLD) differed only in the frontal white matter, with the FTLD group showing higher microglial scores ($p = 0.025$).

Microglial Cell Activation in the Cortical and Subcortical Areas

To assess the distribution of microglial cell activation in each group, the Wilcoxon signed-rank test was used. In the AD group, there were no differences when comparing the microglial scores in the cortical and subcortical white matter in each region ($p > 0.05$). In the FTLD group, the entorhinal, temporal, and frontal regions had significantly higher microglial scores in the white matter compared to their respective gray matter ($p = 0.020$, $p = 0.003$,

Table 1. Clinical status, demographic information, and disease duration

Case	Pathological diagnosis	Gender	Braak stage	PMD	Age at onset, years	Age at death, years
1	AD	M	V–VI	n.a.	58	68
2	AD	M	VI	n.a.	58	70
3	AD	M	V–VI	n.a.	54	59
4	AD	M	V	n.a.	53	66
5	AD	M	VI	96	59	69
6	AD	M	VI	n.a.	55	65
7	AD	F	VI	130	54	61
8	AD	M	V–VI	n.a.	71	76
9	AD	M	V–VI	96	57	66
10	AD	M	VI	96	75	82
11	AD	M	V–VI	61.75	52	65
12	AD	M	V–VI	131	63	70
13	AD	M	V–VI	75	55	64
14	AD	F	VI	64	64	71
15	AD	M	VI	107	64	73
16	FTLD-TDP A	M	n.a.	n.a.	49	58
17	FTLD-TDP A	M	n.a.	n.a.	60	68
18	FTLD-TDP A	M	n.a.	n.a.	59	64
19	FTLD-TDP A	M	n.a.	n.a.	69	75
20	FTLD-TDP A	M	n.a.	n.a.	54	66
21	FTLD-TDP A	M	n.a.	n.a.	64	72
22	FTLD-TDP A	F	n.a.	45.5	66	72
23	FTLD-TDP A	M	n.a.	96	78	82
24	FTLD-TDP A	M	n.a.	94.5	63	65
25	FTLD-TDP A	M	n.a.	81	54	65
26	FTLD-TDP B	M	0–I	n.a.	60	63
27	FTLD-TDP A	M	0	<24	54	61
28	FTLD-TDP C	F	I	6	65	78
29	Control	F	IV	n.a.	n.a.	81
30	Control	F	IV–V	n.a.	n.a.	79
31	Control	F	I–II	72	n.a.	75
32	Control	M	I–II	12	n.a.	85
33	Control	F	0	92.5	n.a.	53
34	Control	M	I–II	48	n.a.	69
35	Control	F	I–II	48	n.a.	60
36	Control	F	I–II	48	n.a.	69
37	Control	M	I	24	n.a.	63
38	Control	F	I	51	n.a.	71
39	Control	F	0	60	n.a.	62
40	Control	M	III	23	n.a.	77
41	Control	F	0–I	21	n.a.	80
42	Control	F	I–II	29	n.a.	79
43	Control	F	0	48	n.a.	81
44	Control	M	0	48	n.a.	68
45	Control	M	I	38	n.a.	56
46	Control	M	I–II	24	n.a.	59

Group	Age at onset[1]	Age at death[1]	Disease duration[1]
AD ($n = 15$)	59.47±6.74	68.33±5.81	8.87±2.56
FTLD ($n = 13$)	61.15±7.66	68.38±6.99	7.23±3.39
Controls ($n = 18$)	n.a.	70.39±9.78	n.a.

n.a., not available.

[1] These values refer to the mean number of years ± SD.

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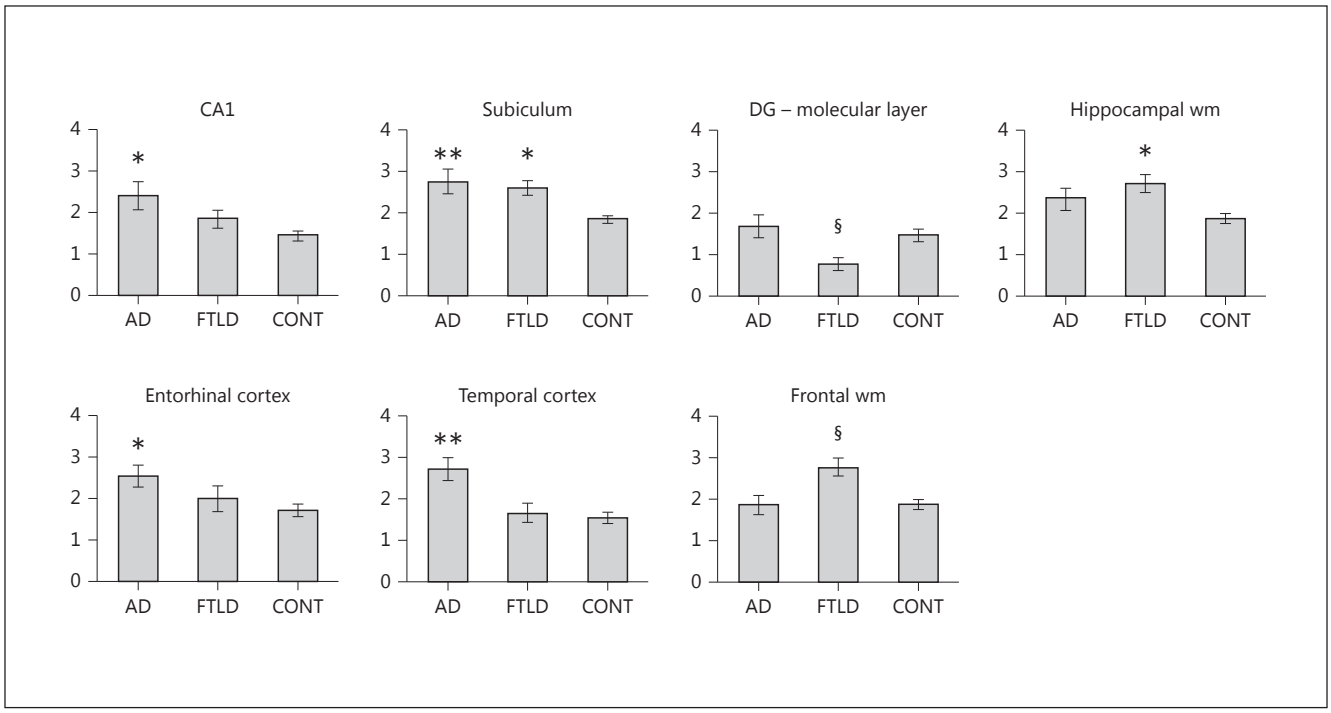

Fig. 3. Comparison of microglia scores between groups in different regions. Bars represent ± SE. CONT, control; DG, dentate gyrus; wm, white matter. * $p < 0.05$ compared to control cases; ** $p < 0.01$ compared to control cases; § $p < 0.05$ compared to control and AD cases.

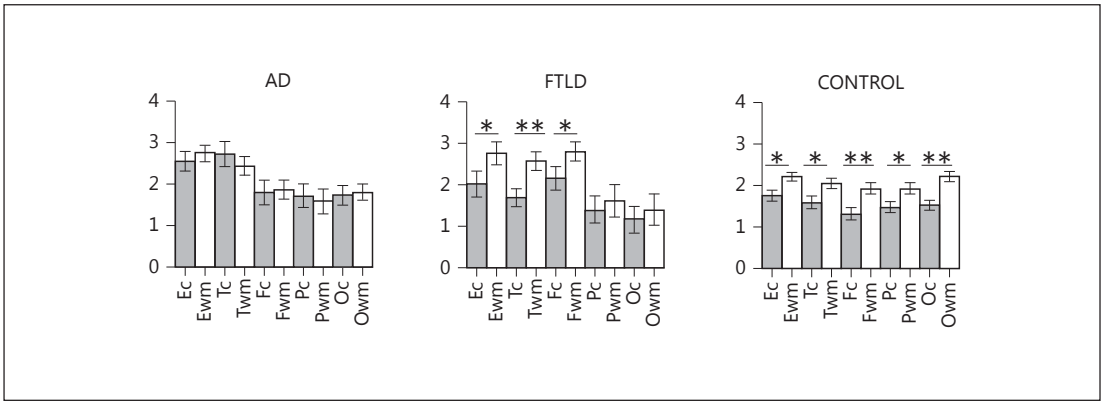

Fig. 4. Comparison of microglia scores between gray matter and white matter within groups in different regions. Ec, entorhinal cortex; Ewm, entorhinal white matter; Tc, temporal cortex; Twm, temporal white matter; Fc, frontal cortex; Fwm, frontal white matter; Pc, parietal cortex; Pwm, parietal white matter; Oc, occipital cortex; Owm, occipital white matter. Bars represent ± SE. * $p < 0.05$; ** $p < 0.01$.

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and $p = 0.021$, respectively). As expected, in the control group, the test showed that all cortical areas had lower microglial scores when compared to their subcortical white matter ($p = 0.011$ for the entorhinal, temporal, and parietal areas; $p = 0.001$ in frontal area and $p = 0.003$ in occipital area) (Fig. 4).

Correlations between Microglial Cell Activation and Age at Onset, Age at Death, and Disease Duration

In the AD group, there was a positive correlation between both age at onset and age at death and the occipital cortex microglial scores ($r = 0.590$, $p = 0.021$, and $r = 0.570$, $p = 0.027$, respectively). For the occipital white matter, there was a positive correlation only with age at death ($r = 0.603$, $p = 0.017$). There were no correlations in any of the other areas studied or with the years of disease duration.

In the FTLD group, there were no correlations between any of the 3 variables and the microglial cell activation scores.

In the control group, there were no correlations between age at death and microglial scores in any region.

Discussion

This study underscores the differences in severity and distribution of activated microglial cells in 2 neurodegenerative dementias with distinct misfolded protein signatures. We assessed and compared levels of microglial cell activation in 3 groups (cases of AD and FTLD-TDP and controls) in several cortical and subcortical white matter areas (entorhinal, temporal, frontal, parietal, and occipital), and also hippocampal formation.

In the hippocampal region, we found higher microglial scores in the CA1 and subiculum in the AD group than in controls. This is not surprising, given that the CA1 and the subiculum region of the hippocampus are the areas the most affected by neuronal loss in AD [20]; they are also associated with a higher burden of Aβ plaques and NFT [21]. Postmortem studies on AD brains and in AD mouse models also show the increase of activated microglial cells in these areas [22–25]. Furthermore, it is known that the density of NFT correlates with that of activated microglia in the subiculum but not in other hippocampal areas [26]. Although microglial activation is a known feature of FTLD pathology [9, 13], the detailed histopathological study of the different hippocampal areas has not been done before.

In this study, we found that the FTLD group showed higher microglial scores than the control group in the subiculum, but not in the CA1 area. Additionally, there was significantly higher microglial activation in the hippocampal white matter in the FTLD than in the control group.

We excluded AD patients with concomitant hippocampal TDP43 pathology, in order to avoid difficulties in the interpretation of the results. Episodic memory deficits are a well-established early feature of AD [27], and there is a widely documented relationship between hippocampal impairment and early episodic memory deficits in AD [28]. Moreover, CA1 pathology (neuron loss and NFT burden) correlates with the memory deficits [29, 30]. Interestingly, some studies have demonstrated that patients with a behavioral variant of FTD can present with severe episodic memory [6, 31], and pathological studies have shown atrophy of the hippocampus [14, 18, 32] and other structures of the Papez circuit [33] on the FTLD spectrum. Additionally, in vivo imaging studies have shown that behavioral-variant FTD cases are associated with more extensive white matter degradation than in AD [34, 35]. Our study supports the contribution of extrahippocampal structures for the episodic memory deficits found in FTLD-TDP patients, i.e., within the connection pathways, as we found prominent microglial activation in the hippocampal white matter (alveolus) without CA1 involvement. This microglial activation is likely to reflect the response to a transsynaptic or transaxonal retrograde cortical degeneration. Moreover, the higher microglia scores found in the DG-ML of the AD group versus in the FTLD group emphasize the contribution of Aβ pathology to the local neuroinflammatory process driven by the microglia. Surprisingly, the control group showed higher microglial scores in this region than the FTLD group; although this finding is difficult to explain (and it is probably related to some Aβ deposits found in the control group), it reinforces the absence of microglial reaction in the DG of the FTLD-TDP cases (despite the known dentate fascia TDP43 inclusions in this pathology).

When addressing the cortex and subcortical white matter regions, and as would be expected, we found higher levels of microglial cell activation in the AD group than in controls in the entorhinal and temporal cortices, 2 key regions affected by AD pathology. In contrast to Lant et al. [13], we did not find differences in the temporal subcortical white matter or the frontal cortex. The differences in the age at death, particularly in the control group (younger and with a wider age range in Lant et al. [13]) in these studies can partially explain this discrepancy. There

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are compelling data that microglia shift towards a pro-inflammatory state with aging [36]. Previous studies have showed that the density of ferritin-stained microglial cells were higher in nondemented elderly control subjects than in young controls in the entorhinal cortex and all areas of the hippocampal formation [26]. Taking this into account, the magnitude of the differences found in the quantification of microglial cell activation, at least using the methods of this study, can be attenuated with increasing aging due to the age-related changes in microglia.

The frontal subcortical white matter in the FTLD group was different from that in the control group, and there was trend of higher microglial activation in the frontal cortex of the FTLD group. Lant et al. [13] also described higher microglial activation scores in the frontal and temporal cortices and the associated subcortical white matter compared to controls. They showed that FTLD-tau have more microglial activation cells in the temporal region (cortical and particularly subcortical white matter) than FTLD-TDP [13]. In our study, we analyzed only the FTLD-TDP subtype, and our results reinforce their finding of a less microglial pathology in its temporal regions. As expected, there were no differences in the other cortical areas analyzed, in agreement with the selective anatomical involvement that characterizes most FTLD cases [37]. Interestingly, when comparing the 2 pathological groups (AD and FTLD), we found the same differences in subcortical frontal gray matter as Lant et al. [13] found in these 2 groups, and the FTLD group showed higher microglial scores, but not in temporal cortex (although there was a trend for higher microglial scores in the AD group). The smaller number FTLD cases in our sample may also have contributed to some of the discrepancies between these 2 studies. However, our findings support the importance of neuroinflammation in neurodegenerative disease, particularly the more pronounced microglial activation in the frontal subcortical white matter in FTLD-TDP and the more prominent involvement of temporal regions in AD. This pattern likely reflects the distribution of the pathology signature of both conditions. In AD, there is a recognized association between microglial cells and amyloid plaques, leading to a higher cortical gray matter-activated microglia burden in this group [38]. In FTLD-TDP, our findings possibly reflect the direct involvement of TDP43 glial cytoplasmic inclusions usually seen in cerebral white matter, or, and more likely, a microglial cell activation response to a transsynaptic or transaxonal retrograde cortical degeneration. The latter concept is supported by the correlation between the extent of microglial activation and neuronal loss but no correlation with TDP-43 pathology, the finding of a pathological autopsy study of patients with amyotrophic lateral sclerosis with and without dementia [39].

Interestingly, regional analysis showed the prominent involvement of the white matter rather than the regional cortical gray matter in the FTLD group, namely, in the entorhinal, temporal, and frontal regions. In the AD group, despite there being more frequent clusters of activated microglia surrounding the amyloid plaques in the cortical gray matter, there were no differences when this was compared the subcortical regional white matter. These findings reflect the presence of a more diffuse microglial activation pathology in end-stage AD brains, and reinforce the imaging studies that showed more extensive white matter degradation in the behavioral variant of FTD than in AD [34, 35]. As expected, according to microglia distribution in normal human brains [36], all cortical areas had lower microglial scores than the regional subcortical white matter in the control group.

The findings reported in this study should be interpreted by taking into account the possibility of interindividual variability in the pathology burden of the 2 entities (Aβ deposition, tau, and TDP pathology), and further studies that correlate microglial activation with these pathology markers would be helpful. For instances, a recent study reported that microglial cells in the hippocampi of severely affected Braak stage V–VI samples were no longer even associated with neuritic plaques or with the vascular amyloid, highlighting the range of microglial alterations [40].

Despite the relatively small age range of the patients in this study, we found a positive correlation between age and microglial activation scores in the occipital cortex of the AD group. This should be further analyzed in a bigger sample with a greater age range, but it does highlight the possibility of the age-modifying effect in microglia reactions in AD. Vascular pathology was not significant in either of the groups (there was minor small-vessel disease in 1 AD case and 1 control case), so this does not explain differences.

In conclusion, our work extends the knowledge of the distribution and magnitude of microglial activation in these 2 conditions, and describes some microglial circuit-specific patterns that can help to explain the clinical overlap between AD and FTLD-TDP, namely, in memory deficits.

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Statement of Ethics

This work was approved by the Ethics Committee of the Portuguese Brain Bank – Centro Hospitalar do Porto.

Disclosure Statement

There are no conflicts of interest to report relative to this work by any of the authors.

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3.1.2 Analysis of pathological neuroinflammatory markers (microglia and astrocytes) in clinically and pathologically confirmed AD patients with different disease ages of onset

Taipa R, Ferreira V, Brochado P, Robinson A, Reis I, Marques F, Mann DM, Melo-Pires M, Sousa N

Inflammatory pathology markers (activated microglia and reactive astrocytes) in early and late onset Alzheimer disease: a post-mortem study

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Inflammatory pathology markers (activated microglia and reactive astrocytes) in early and late onset Alzheimer disease: a *post mortem* study

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Inflammatory pathology markers (activated microglia and reactive astrocytes) in early and late onset Alzheimer disease: a *post mortem* study

Aims: The association between the pathological features of AD and dementia is stronger in younger old persons than in older old persons suggesting that additional factors are involved in the clinical expression of dementia in the oldest old. Cumulative data suggests that neuroinflammation plays a prominent role in Alzheimer's disease (AD) and different studies reported an age-associated dysregulation of the neuroimmune system. Consequently, we sought to characterize the pattern of microglial cell activation and astrogliosis in brain *post mortem* tissue of pathologically confirmed cases of early and late onset AD (EOAD and LOAD) and determine their relation to age. **Methods**: Immunohistochemistry (CD68 and glial fibrillary acidic protein) with morphometric analysis of astroglial profiles in 36 cases of AD and 28 similarly aged controls. **Results**: Both EOAD and LOAD groups had higher microglial scores in CA1, entorhinal and temporal cortices, and higher astroglial response in CA1, dentate gyrus, entorhinal and temporal cortices, compared to aged matched controls. Additionally, EOAD had higher microglial scores in subiculum, entorhinal and temporal subcortical white matter, and LOAD higher astrogliosis in CA2 region. **Conclusions**: Overall, we found that the neuroinflammatory pathological markers in late stage AD human tissue to have a similar pattern in both EOAD and LOAD, though the severity of the pathological markers in the younger group was higher. Understanding the age effect in AD will be important when testing modifying agents that act on the neuroinflammation.

Keywords: ageing, Alzheimer's disease, astrocytes, microglia, pathology

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder and is the most common cause of dementia. The two major neuropathological hallmarks of the disease are senile plaques, mainly composed of extracellular deposits of amyloid β (Aβ), and neurofibrillary tangles, consisting of intracellular aggregates of

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aberrantly phosphorylated tau protein. This is accompanied by neuronal and synaptic loss, dendritic and axonal changes and inflammatory reactive lesions [1,2]. Cumulative data suggest that neuroinflammation plays a prominent and early role in AD [3–9]. In fact, neuropathological studies have shown the presence of a broad variety of inflammation-related proteins (complement factors, acute-phase proteins, proinflammatory cytokines) and clusters of activated microglia around amyloid plaques in both transgenic models and AD subjects [8]. Furthermore, polymorphisms in genes encoding microglia-specific proteins involved in phagocytic and protein degradation pathways increase the risk of AD [10,11]. Reactive astrocytes also tend to accumulate around fibrillar amyloid plaques [12] and similar to microglia, astrocytes release cytokines and other potentially cytotoxic molecules after exposure to Aβ, thus aggravating the neuroinflammatory response [9].

The prevalence of AD is strongly associated with increasing age, and age-related changes in microglia have been hypothesized to play a prominent role in disease pathogenesis [13]. Regardless of clinical resemblance and neuropathological features, important differences exist between early and late onset AD [EOAD (<65 years old) and LOAD (>65 years old)] patients [14–17]. Taking into account data regarding the importance of neuroinflammation in the pathogenesis of AD, particularly the role of microglia, and the differences in the neuroimmunological milieu of the aged brain, it is conceivable that the pattern of neuroinflammation associated with AD might differ between these two groups and contribute to, or explain, clinical differences [17]. Hoozemans *et al.* [18] suggested that an association between neuroinflammation and AD is much stronger in relatively young patients compared to older patients (age at death <80 *vs.* >80 years old). More recently, shortening of microglial cell processes and reduced of coverage of brain parenchyma with normal ageing and AD has been reported [19].

In this study, we characterized the distribution of activated microglia in multiple anatomical areas (hippocampal formation; frontal, temporal, parietal and occipital cortical grey and subcortical white matter) and the degree of astrogliosis in selected areas (hippocampal formation and temporal cortical grey and subcortical white matter), in clinically and pathologically confirmed AD and non-demented control cases in relation to age. Our results suggest that overall there is a similar topographical pattern in pathological markers of neuroinflammation in both EOAD and LOAD. However, there are differences in the severity of microglial scores and astrogliosis when comparing these two groups with age-matched controls.

Patient and methods

Patients

Fifty-seven cases were investigated. The majority of cases were obtained from the Manchester Brain Bank (30 AD and 9 control cases) through appropriate consenting procedures for the collection and use of the human brain tissues. Additional control cases were obtained from Oxford Brain Bank (14 cases) and from Queen Square Brain Bank (4 cases), also through appropriate consenting procedures for the collection and use of the human brain tissues. Cases were approximately age-matched. The AD cases met the pathological criteria for definite AD ('High' AD neuropathologic change) [20]. Cases with associated hippocampal sclerosis were excluded. The 18 controls were judged to be clinically normal and none showed any pathology beyond that which might be anticipated for age. The study was approved by the relevant local Brain Bank Committees under their devolved Generic Tissue Bank ethics.

Methods

Immunohistochemistry (IHC) IHC staining for CD68 and glial fibrillary acidic protein (GFAP) was performed using the Ventana OptiView DAB IHC detection kit and the Ventana BenchMark Ultra processor (Ventana, Tucson, AZ, USA). Sections of temporal (including hippocampus and parahippocampal region), frontal, parietal and occipital cortices were cut at 6 μm thickness from formalin fixed, paraffin embedded blocks and mounted on to glass slides. Paraffin tissue sections were deparaffinized with EZ Prep (Ventana), pre-treated with heat treatment with Ultra Cell Conditioning Solution (CC1; Ventana) and the endogenous peroxidase was inactivated before the incubation with the CD68 antibody (PG-M1, 1:400; Dako, Glostrup, Denmark) for 24 min at 36°C and GFAP (Z 0334,

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1:2500; Dako) for 12 min at 36°C. Subsequently, the slides were incubated with OptiView HQ linker for 8 min, and then with OptiView universal HRP multimer (Ventana) for a further 8 min at 36°C. Tissue sections were incubated with OptiView universal DAB chromogen (Ventana) for 8 min to detect the antigen-antibody complex and then counterstained with Haematoxylin II (Ventana).

Similar study methods for IHC were used with Iba1 antibody (Wako Cat. #019-19741, Wako Pure Chemical Industries, Osaka, Japan) using a series of random temporal cortical sections across the groups (16 cases).

Microscopic semi-quantitative analysis Microglia—Both CD68 and Iba1 antibodies mark microglial cells, both in resting and activated states (Figure 1**a**,**b**). Resting microglia were defined by a small cell body and ramified processes [21] and activated microglia by their shortened processes and hypertrophy of cell body [22]. Sections from the different interest regions were assessed for the presence of immunostained microglial cells within both the cortical grey and subcortical white matter at × 20 magnification. As previously described [23,24], the frequency and 'severity' (in terms of morphological types, with activated microglial cells being considered to be more severe than ramified microglial cells) of CD68-immunostained sections was assessed according to:

0 = No immunostained cells present.

1 = Very few immunostained cells present, all as ramified microglia.

2 = A moderate number of immunostained cells present, mostly ramified but some activated cells present.

3 = Many, diffusely spread, immunostained cells present, all as activated microglia.

4 = Many, large clusters of activated microglial cells present (Figure 2).

Perivascular CD68 immunostained cells (perivascular macrophages) were discounted for the scoring analysis. For each section, the cortex and white matter was scored separately. Additionally, in the temporal block, hippocampal formation [CA1, CA2/3, dentate gyrus

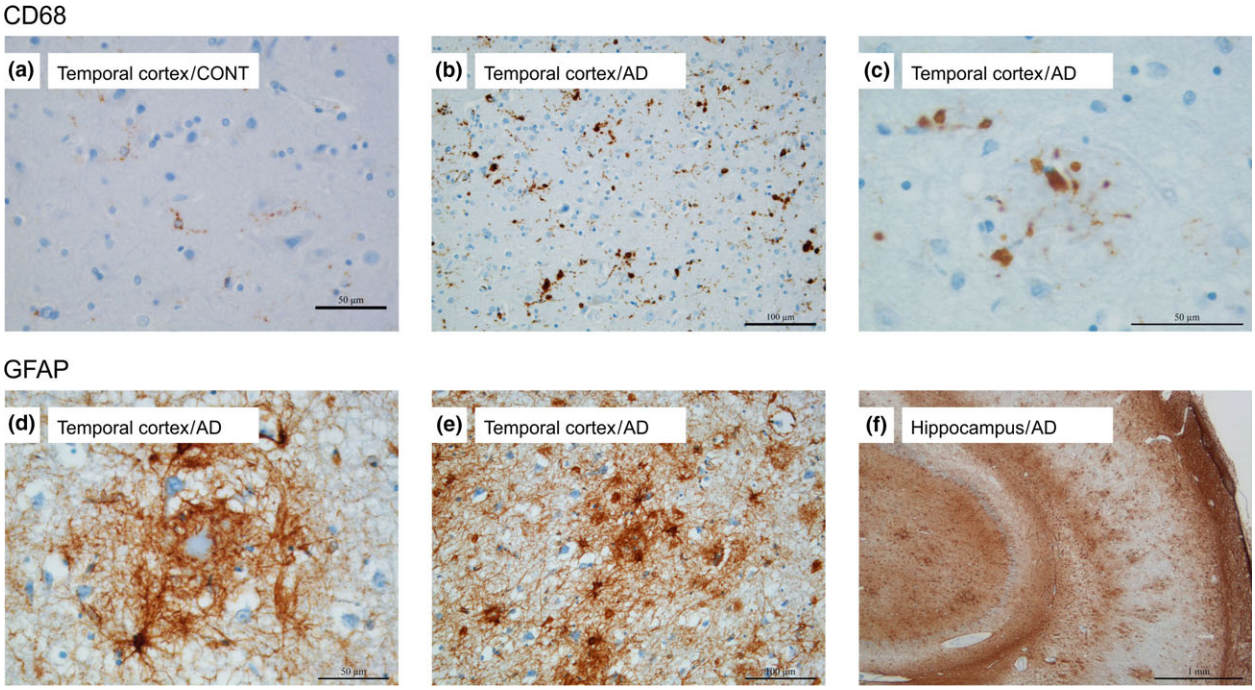

Figure 1. Ramified (**a**) and activated (**b**) microglia cells, as seen in CD68 immunostaining in temporal cortex [**a** – CONTy case; **b** – early onset Alzheimer's disease (EOAD) case]. Clustering of activated microglial cells within an amyloid plaque (**c**, EOAD case). Astrocytes immunoreactive for glial fibrillary acidic protein (GFAP) immunostaining closely associated to an amyloid plaque (**d**) and dispersed in the cortex (**e**). Example of a hippocampal section of an AD case with GFAP immunostaining (**f**). (**d**–**f**, EOAD case). Scale bars: 50 μm (**a**,**c**,**d**), 100 μm (**b**,**e**), 1 mm (**f**). **a**–**c**: CD68 immunohistochemistry (PGM1 clone; Dako); **d**–**f**: GFAP immunohistochemistry (Z 0334; Dako).

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(DG) and subiculum], hippocampal white matter, entorhinal cortex and entorhinal white matter were also scored separately.

The assessment of Iba1 scores followed the same procedures as CD68.

Astrocytes—Astrogliosis is characterized by cellular hypertrophy with an increase in expression of GFAP and an abnormal apparent increase in the number of astrocytes [25]. Sections of the temporal block were assessed for the presence of astrogliosis within both the cortical grey and subcortical white matter and hippocampal formation. The presence and degree of astrogliosis of GFAP-immunostained sections was assessed according to:

0 = No GFAP immunostained cells present.

1 = Very few GFAP immunostained cells present with thinly ramified processes and no cell body hypertrophy.

2 = Moderate number of GFAP immunostained cells present, some with intense immunoreactive processes and cell body hypertrophy.

3 = Many, diffusely spread, GFAP immunostained cells present, all with cell body hypertrophy and intense immunoreactive processes.

4 = Many GFAP immunostained cells present, all with cell body hypertrophy and intense immunoreactive processes with areas of large clusters of GFAP immunostained cells with these characteristics (Figure 2).

In the white matter, increased numbers of fibroblastic astrocytes were employed for grading (0–3) with increased GFAP staining with aspects of glial scarring being considered for grade 4.

All microglial and astrocytes assessments were made by a single observer (RT), who was blinded to case diagnostics.

Cell counting procedure An unbiased microscopic stereological analysis was performed in the subiculum and entorhinal cortex (five cases per group) and correlated with the semi-quantitative scales scores for microglia activation and astrogliosis. Quantification of $GFAP^+$ and $CD68^+$ cells was performed in accordance with the following criteria: (i) large cells with increased processes complexity were counted as $GFAP^+$ cells; and (ii) large cells with shortened processes were counted as $CD68^+$ cells (activated microglia), respectively. Additionally, a total count of cells with visible processes (small and thick or ramified) in CD68 immunohistochemistry assay was performed. The cells were counted on Visiopharm Integrator System Software (version 2.12.3.0; Hoersholm, Denmark), using a motorized microscope (BX-51; Olympus, Hamburg, Germany) attached to a digital camera (U-TV1X-2; Olympus), with the 40× oil-immersion objective. The subiculum and entorhinal cortex were selected and inside it, two smaller areas were designated for cell counting. Square probes (50 × 50 μm)

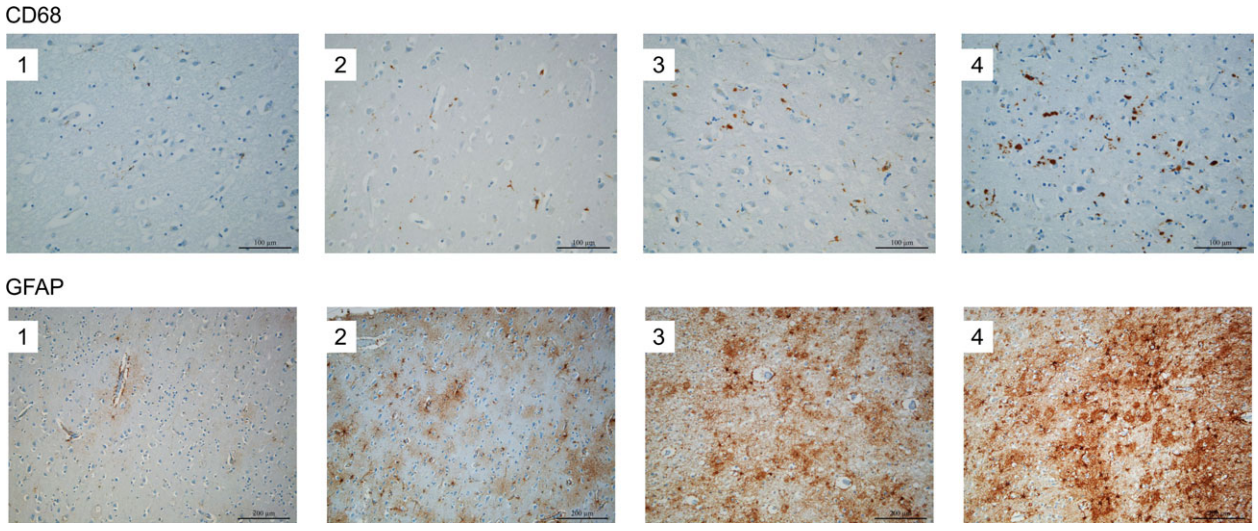

Figure 2. Representative examples of the microglial and astrocytosis assessment according to the scale description. Scale bars: 100 μm (CD68) and 200 μm [glial fibrillary acidic protein (GFAP)]. CD68 (PGM1 clone; Dako) and GFAP immunohistochemistry (Z 0334; Dako).

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were placed randomly over the selected areas, covering 20% of the total defined areas, ensuring unbiased sampling. The cells within the criteria aforementioned and inside the probes were counted. All counts were performed by a single observer (VF) blinded to the diagnosis.

Astrocytes profiles morphological analysis Three-dimensional reconstructions of representative $GFAP^+$ cells within the subiculum region were made. Cell reconstruction was performed in accordance with the following criteria: (i) dendritic tree does not have truncated processes and (ii) relative isolation from neighbouring marked cells. According these criteria and using the Neurolucida software (MBF Bioscience, Williston, VT, USA) and a motorized microscope (Axioplan 2; Carl Zeiss, Oberkochen, Germany) attached to a camera (3CCD Color Video Camera; Sony, Minato, Tokyo, Japan), the $GFAP^+$ cells were reconstructed, with the 100× oil-immersion objective. The first three $GFAP^+$ cells identified in the region of interest that complied with the criteria were reconstructed, per experimental case, and subsequently analysed by NeuroExplorer Software (MBF Bioscience). All reconstructions were performed by a single observer (VF) blinded to diagnosis.

Statistical analysis

Rating data was entered into an Excel spreadsheet and analysed using Statistical Package for Social Sciences (SPSS) software (version 22.0), Armonk, NY. A *P*-value of <0.05 was considered statistically significant. Kruskal–Wallis test was performed for analysis. If this detected any significant differences between the groups, *post hoc* testing was performed to identify the groups between which significant differences existed (Dunn–Bonferroni). Kendall's τ coefficient was used to assess correlation between age and microglial scores or astrogliosis in each group.

Results

Four groups were established (Table 1) based on confirmation from pathology (AD *vs.* controls) and age at death. In the AD group, a cut-off of ≤ 65 years for age of onset was used for classification in EOAD and LOAD. An age at death of 75 years was used in the control group (CONTy and CONTo) as a cut-off to match the age at death of the AD groups. Cases were grouped as EOAD ($n = 19$), LOAD ($n = 11$), young non-demented controls (CONTy) ($n = 15$) and old non-demented controls (CONTo) ($n = 12$). There were no differences between EOAD and CONTy, and between LOAD and CONTo in age at death ($P = 0.575$ and $P = 1.0$, respectively; Kruskal–Wallis). EOAD and LOAD groups differed in the disease duration ($P = 0.016$; Kruskal–Wallis), with EOAD cases having a longer disease duration than LOAD cases (9.8 years *vs.* 7.6 years, respectively). The groups were not homogeneous regarding to gender with over representation of males in EOAD group (see Table S1 for detailed pathological and demographic details, and disease duration per case).

As previously described [23,24], the topographic distribution of activated microglial cells generally followed that of the principal pathological changes within the cortex and hippocampal formation of AD cases, with activated microglial cells often being clustered within and around amyloid plaques (Figure 1**c**). Similarly to other descriptions, activated microglial cells were also

Table 1. Clinical and pathological status, demographic information and disease duration

	Male: female	*Mean age at death (SD)*	*Interval of age at death*	*Mean age of disease onset (SD)*	*Mean disease duration (SD)*
EOAD $n = 19$	17M: 2F	66.74 (±6.62)	45–73	56.72 (±6.11)	9.78 (±2.46)
LOAD $n = 11$	4M: 7F	82.91 (±5.13)	76–89	76.0 (±4.59)	7.60 (±1.26)
Controls young $n = 15$	7M: 8F	59.47 (±8.73)	38–71	NA	NA
Controls old $n = 12$	6M: 6F	83.75 (±6.81)	75–95	NA	NA

NA, not applicable; EOAD, early onset Alzheimer's disease; LOAD, late onset AD.

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observed apparently unassociated with amyloid plaques [26]. In the control cases activated microglial cells were largely absent in most of the cases, though a few were occasionally seen in association with rare amyloid deposits. Ramified microglial cells were commonly seen in many of these cases. The morphological patterns were similar with both antibodies, though Iba1 showed a better definition of the ramified morphology of the resting microglia (Figure S1). CD68 and Iba1 scores showed a strong correlation in the same region analysed ($r = 0.659$, $P < 0.01$ in entorhinal cortex; $r = 0.464$, $P < 0.05$ in entorhinal subcortical white matter, $r = 0.782$, $P < 0.001$ in temporal cortex; $r = 0.529$, $P < 0.05$ in temporal subcortical white matter, Kendall's τ coefficient; Figure S2).

As expected, astrogliosis in the AD group was more prominent in layers II–III and layer V [27]. Similarly to microglia, we found a dense astrogliosis both intimately associated with amyloid plaques and also remote from amyloid plaques [28] (Figure 1**d**,**e**). Most control cases showed rare immunoreactive astrocytes with long and thin dendritic arborizations without laminar pattern. Figure 3 shows representative examples of CD68 and GFAP immunostaining in the entorhinal region in the four different groups.

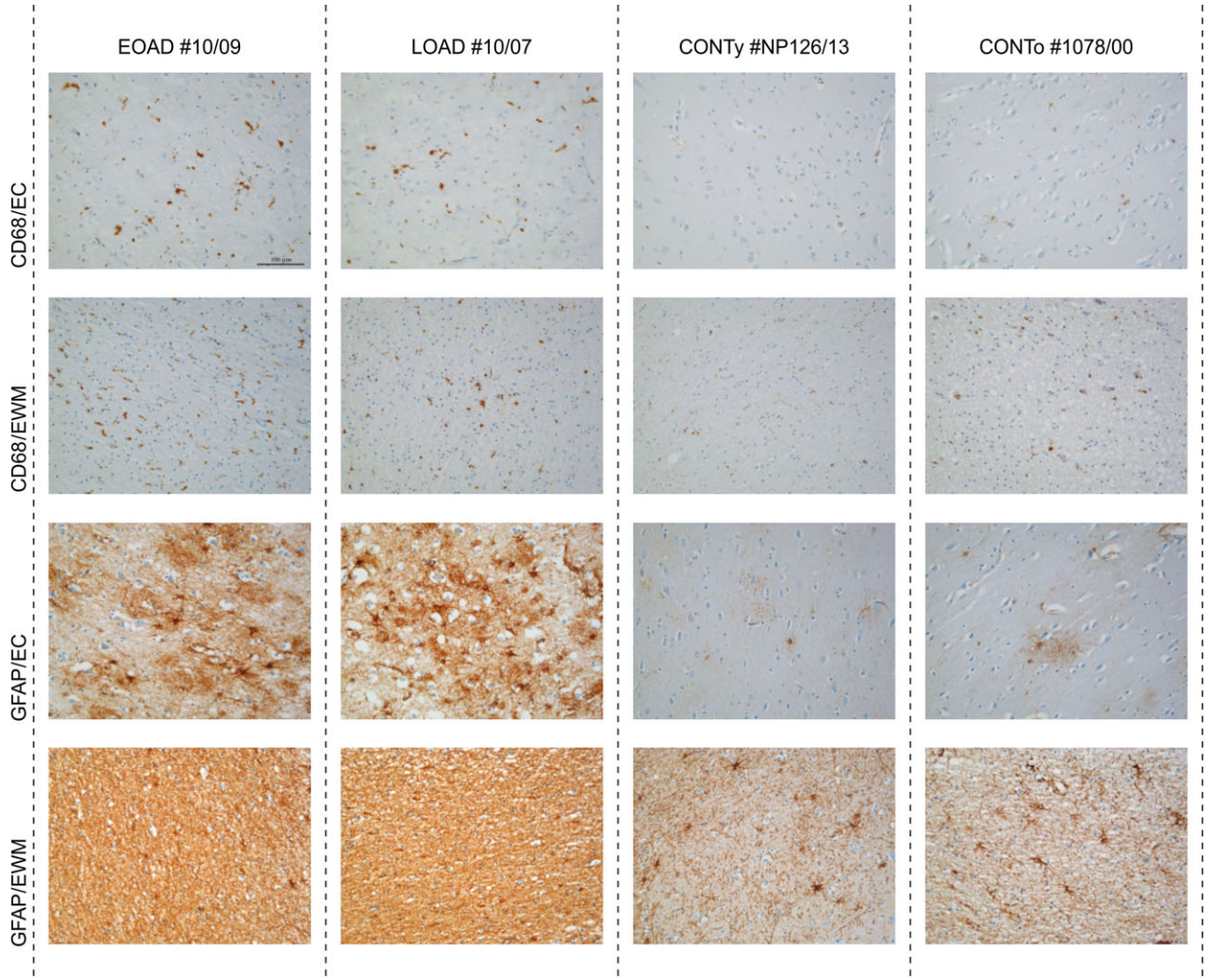

Figure 3. Representative examples of CD68 and glial fibrillary acidic protein (GFAP) immunostaining in cases of the different groups studied. Scale bar: 50 μm. EC, entorhinal cortex; EWM, entorhinal white matter. CD68 (PGM1 clone; Dako) and GFAP immunohistochemistry (Z 0334; Dako).

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Semi-quantitative scores for microglial cell counts showed a strong correlation with stereological counting of activated microglia in the subiculum ($r = 0.629$, $P < 0.001$) and entorhinal cortex ($r = 0.771$, $P < 0.001$). Similarly, semi-quantitative scores for astrogliosis showed a strong correlation with stereological counting of GFAP positive astrocytes in subiculum ($r = 0.629$, $P < 0.001$) and entorhinal cortex ($r = 0.650$, $P < 0.01$) (Figure S3). Interestingly, in the subiculum stereological counting of microglia correlated with GFAP (stereological counting and semi-quantitative scale) only when activated microglia were considered ($r = 0.386$, $P < 0.05$ and $r = 0.471$, $P < 0.05$, respectively).

Microglial scores based on the patterns of CD68 staining

Hippocampal formation Microglial cell scores were significantly different between EOAD, LOAD, CONTy and CONTo groups for the CA1 region of hippocampus ($P = 0.001$), subiculum ($P = 0.001$) and hippocampal white matter ($P = 0.022$). *Post hoc* testing showed that in the CA1 region the AD groups differed from their aged

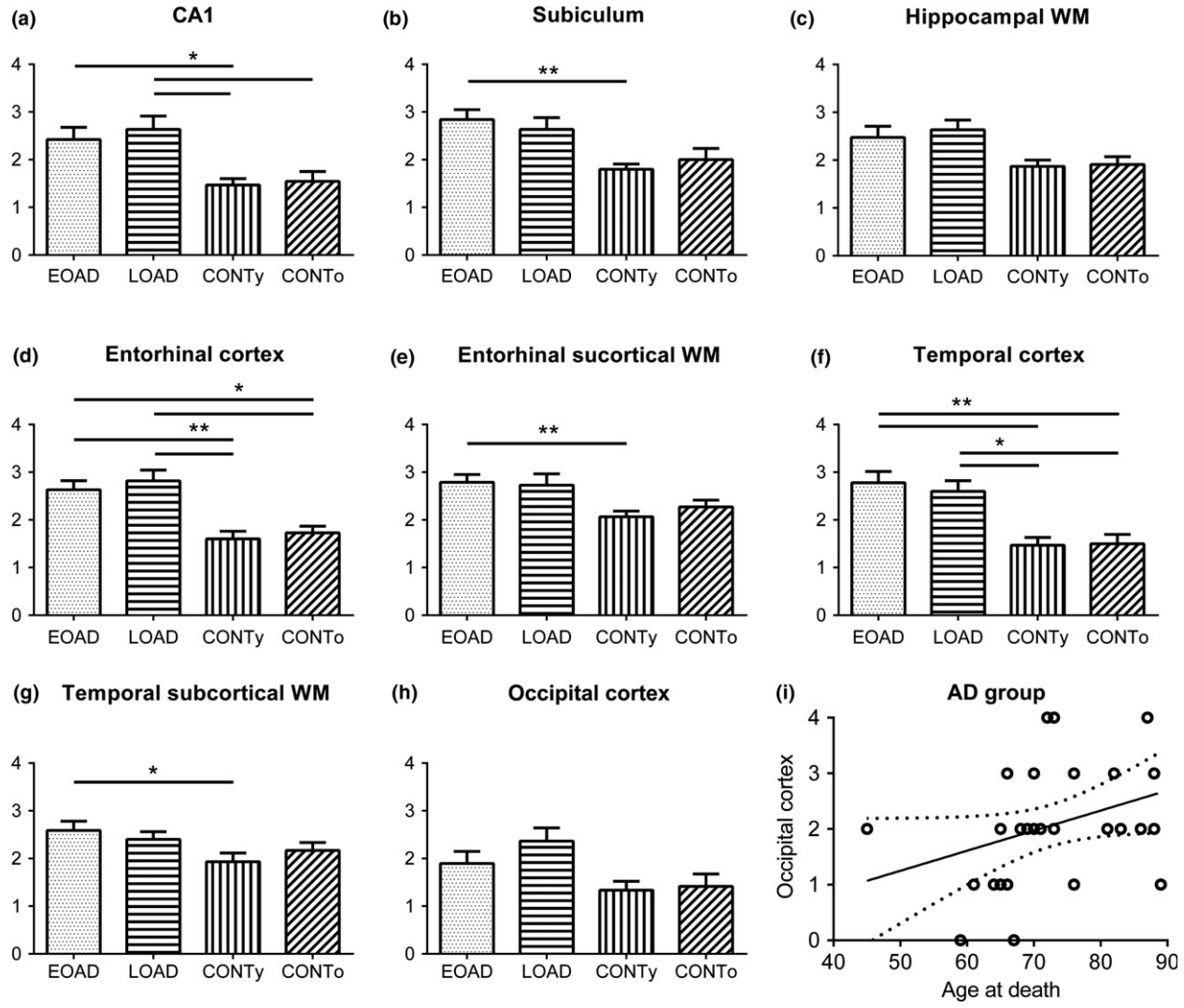

Figure 4. Comparison of microglia scores between groups in different regions (a - h) and correlation between microglia severity score in occipital cortex and age at death (i). a - h: *$P < 0.05$ and **$P < 0.01$, Kruskal–Wallis. *Y* axes represent semi-quantitative scale mean and bars represent ±SE. i: $r = 0.322$; $p < 0.05$, Kendall's t coefficient

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matched controls (EOAD *vs.* CONTy and LOAD *vs.* CONTo, $P = 0.024$ and $P = 0.043$, respectively). Additionally, the LOAD group displayed higher microglial scores than the CONTy ($P = 0.012$) (Figure 4**a**). In the subiculum, only the EOAD had higher microglial scores than their aged matched control group (CONTy) ($P = 0.001$) (Figure 4**b**). *Post hoc* testing in the hippocampal white matter did not show differences between any of the groups (Figure 4**c**). There were no differences between EOAD and LOAD in any of the regions studies. Similarly, no differences were found between CONTy and CONTo groups.

Cortex and subcortical white matter Microglial cell scores were significantly different between EOAD, LOAD, CONTy and CONTo groups for entorhinal cortex and entorhinal subcortical white matter ($P < 0.001$ and $P = 0.005$, respectively) temporal cortex and temporal subcortical white matter ($P < 0.001$ and $P = 0.048$, respectively) and occipital cortex ($P = 0.037$). There was no significant effect of disease status in the other regions studied (frontal cortex and white matter, parietal cortex and white matter, occipital white matter).

Post hoc testing showed that both AD groups had higher microglial scores than their aged matched control groups in entorhinal cortex ($P = 0.002$ for EOAD *vs.* CONTy and $P = 0.018$ for LOAD *vs.* CONTo). They both differed from the non-age matched control group (EOAD *vs.* CONTo, $P = 0.026$; LOAD *vs.* CONTy, $P = 0.002$) (Figure 4**d**). In the entorhinal subcortical white matter, only the EOAD had higher microglia scores compared to the age matched control group ($P = 0.006$). The LOAD did not differ in this region from either of the control groups (Figure 4**e**).

In the temporal cortex, both AD groups had higher microglial scores than their aged matched control groups ($P = 0.001$ for EOAD *vs.* CONTy and $P = 0.028$ for LOAD *vs.* CONTo). Similar to the entorhinal region, they also both differed from the non-age matched control group (EOAD *vs.* CONTo, $P = 0.003$; LOAD *vs.* CONTy, $P = 0.020$) (Figure 4**f**). Similar to the entorhinal region, in the temporal subcortical white matter, only the EOAD showed higher microglial scores compared to their control group (EOAD *vs.* CONT y, $P = 0.045$) (Figure 4**g**). *Post hoc* testing did not show differences between any of the groups in the occipital cortex (Figure 4**h**).

Correlations between microglial cell activation with age of onset, age at death and disease duration In the AD group, there was a positive correlation between age at death and occipital cortex microglial scores ($r = 0.322$, $P = 0.025$) (Figure 4**i**). In addition, in the AD group there was a positive correlation between disease duration and the microglial scores of frontal and occipital white matter ($r = 0.375$, $P = 0.019$; $r = 0.318$, $P = 0.044$, respectively).

In the control group, there was no correlation between age at death and microglial scores.

Astrogliosis based on the patterns of GFAP staining

Hippocampal formation Astrogliosis scores were significantly different between EOAD, LOAD, CONTy and CONTo groups in all hippocampal regions assessed, namely CA1 ($P < 0.001$), CA2/3 ($P = 0.001$), CA4 ($P = 0.012$), DG ($P < 0.001$), subiculum ($P < 0.001$) and hippocampal white matter ($P = 0.002$).

Post hoc testing showed that in the CA1 region, both AD groups had higher astrogliosis scores than their aged matched controls (EOAD *vs.* CONTy, $P < 0.001$ and LOAD *vs.* CONTo, $P = 0.007$) and non-aged matched control group (EOAD *vs.* CONTo, $P = 0.013$ and LOAD *vs.* CONTy, $P < 0.001$) (Figure 5**a**). Remarkably, in CA2/CA3 region only the LOAD group had higher scores compared to both their age matched control ($P = 0.002$) and non-aged matched control ($P = 0.009$) (Figure 5**b**). In CA4 *post hoc* testing did not show differences between any of the groups (Figure 5**c**). In the DG and subiculum both groups differed from their age matched control (EOAD *vs.* CONTy, $P = 0.003$ and LOAD *vs.* CONTo, $P = 0.008$ for DG; EOAD *vs.* CONTy, $P = 0.001$ and LOAD *vs.* CONTo, $P = 0.001$ for subiculum) and non-age matched control (EOAD *vs.* CONTo, $P = 0.005$ and LOAD *vs.* CONTy, $P = 0.005$ for DG; EOAD *vs.* CONTo, $P = 0.001$ and LOAD *vs.* CONTy, $P = 0.001$ for subiculum) (Figure 5**d**,**e**). In the hippocampal white matter, there were differences between EOAD group and CONTy, with higher scores in the former group ($P = 0.003$). LOAD showed higher scores only when compared to the non-aged matched control (LOAD *vs.* CONTy, $P < 0.001$).

Entorhinal and temporal neocortical regions Astroglial scores were significantly different between EOAD, LOAD, CONTy and CONTo groups for entorhinal cortex

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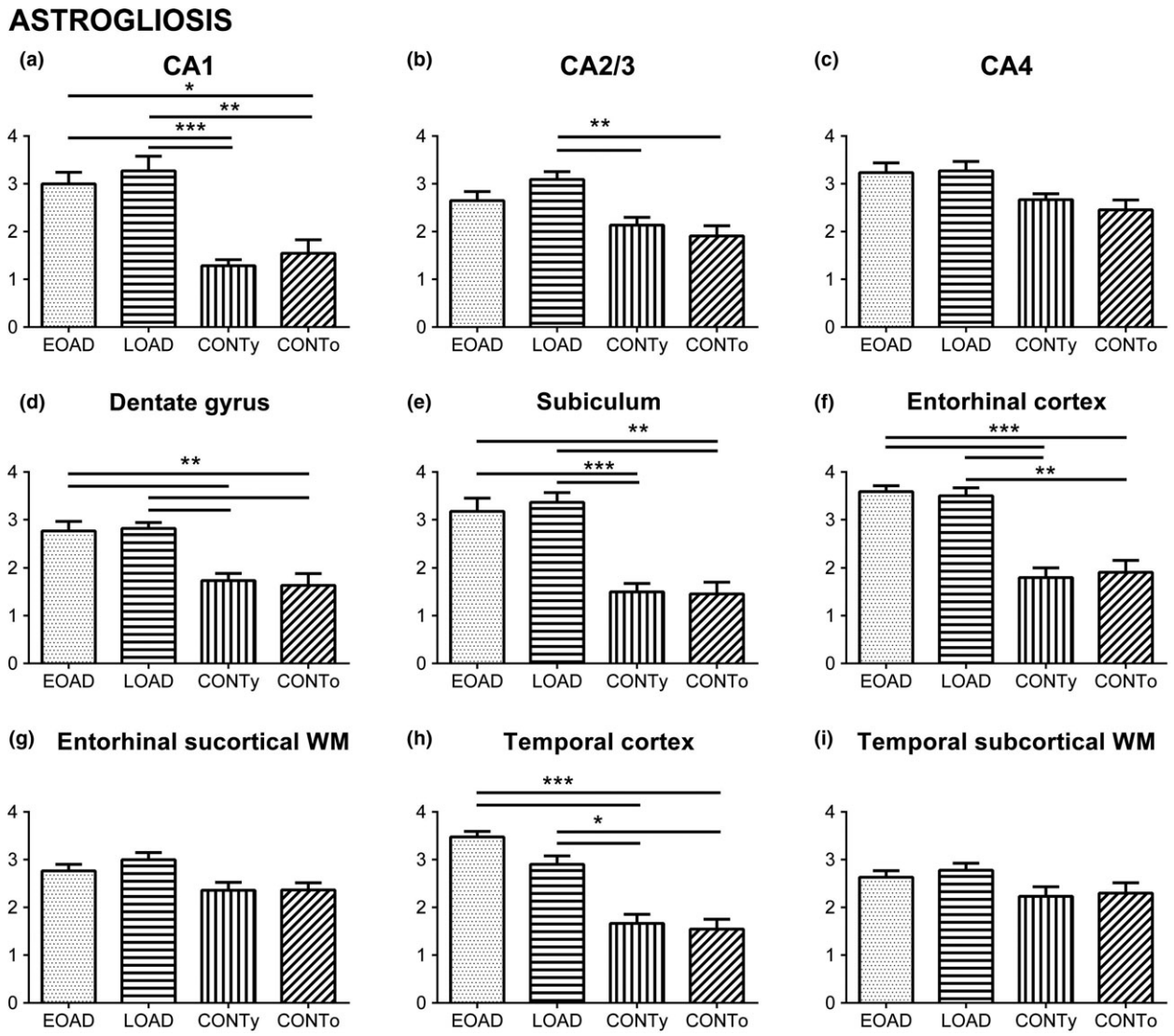

Figure 5. Comparison of astroglial scores between groups in different regions (a - i). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, Kruskal–Wallis. *Y* axes represent semi-quantitative scale mean and bars represent ±SE.

and temporal cortex ($P < 0.001$ for both) and entorhinal subcortical white matter ($P = 0.022$). There was no significant effect of disease status in the temporal cortex white matter.

Post hoc testing showed that in the entorhinal and temporal cortices, both groups differed from their age matched control (EOAD *vs.* CONTy, $P < 0.001$ and LOAD *vs.* CONTo, $P = 0.004$ for entorhinal cortex; EOAD *vs.* CONTy, $P < 0.001$ and LOAD *vs.* CONTo, $P = 0.021$ for temporal cortex) and non-age matched control (EOAD *vs.* CONTo, $P < 0.001$; LOAD *vs.* CONTy, $P = 0.001$ for entorhinal cortex; EOAD *vs.* CONTo, $P < 0.001$ and LOAD *vs.* CONTy, $P = 0.025$ for temporal cortex). In the entorhinal subcortical white matter, *post hoc* testing did not show differences between any of the groups (Figure 5**f**–**i**).

Correlations between and astrogliosis with age of onset, age at death and disease duration In the AD group, there was a positive correlation between age at death and astrogliosis scores in the hippocampal white matter ($r = 0.393$, $P = 0.039$) and entorhinal cortex

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subcortical white matter ($r = 0.392$, $P = 0.043$). There was no correlation with other regions or with age of onset or disease duration.

In the control group, there was a positive correlation with age at death and hippocampal white matter ($r = 0.450$, $P = 0.006$). There was no correlation with other regions studied.

Astroglial morphological profiles

Forty cases showed sufficient quality of immunohistochemistry staining to permit morphological analysis (13 EOAD, 5 LOAD, 13 CONTy and 9 CONTo). There were no significant differences in surface, volume or cell body area and perimeter of astrocytes in the subiculum between the different groups. Similarly, when considered together there were no differences between the AD group (EOAD plus LOAD) and controls (Figure 6).

Discussion

There is clinical and experimental evidence that the aged brain is characterized by a shift towards a pro-inflammatory state [29,30]. This age-associated neuroinflammation is characterized by an upregulated

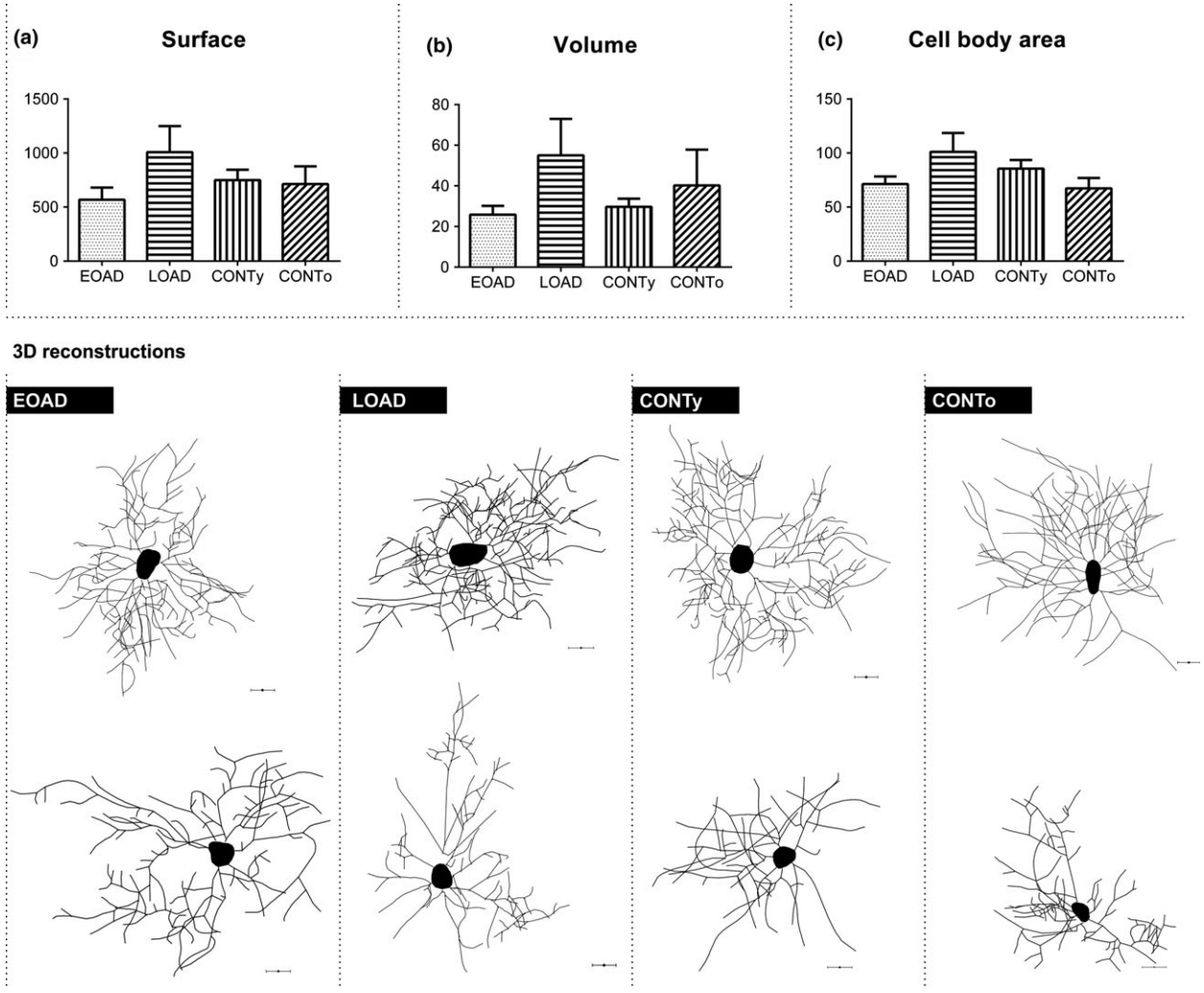

Figure 6. Bar graphs representing glial fibrillary acidic protein surface, volume, and cell body area of astrocytes in subiculum (a - c; *Y* axes represent cell count density in μm^2). Representative examples of astrocyte profiles reconstructions in the bottom panel. Variability within groups is depicted by astrocytes with higher and lower complexity of processes (upper and lower examples within groups respectively). Scale bar: 10 μm

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astrocyte and microglial cell reactivity in association with increased levels of circulating cytokines such as TNF-alfa, IL-1beta and IFN-gamma [31,32]. AD is an age-related disease and there is compelling data showing that the activation of the immune system accompanies and contributes to the pathogenesis of this disorder [33,34]. There is still ongoing controversy about the role of neuroinflammation in AD, namely the possibility of a disease-initiating mechanism in neurodegenerative disorders [34]. Large numbers of studies have investigated the microglia and astrocyte response to Aβ, mainly using *in vitro* culture approaches and/or animal models for tissue immunostaining. However, and as an example, the study of AD degenerative process involving microglial cells in the AD hippocampus of human *post mortem* samples is not mimicked by Aβ models, and only partially so by tau animal models [26]. Human *post mortem* studies are relatively few and, despite their inherent difficulties, their major value is the study of the disease itself and, thus, helping in bridging the vast knowledge obtained by disease animal models.

This study explores the severity of neuroinflammation pathology markers (microglia activation and astrogliosis) in AD pathologically proven cases (Braak stage V–VI) and controls in relation to their age. Two populations were defined according to the age onset/age of death to mimic clinical common distinction between EOAD and LOAD. Our results show that overall the pattern of neuroinflammation is similar between both age AD groups. In fact, we did not find the differences reported by Hoozemans *et al.* [18] claiming a stronger association with neuroinflammation in relatively young AD cases compared to old AD cases. A major difference between the two studies relates to the pathological criteria for inclusion: while in the present study Braak stage V or VI was a pathological criterion for inclusion, in the Hoozemans *et al.* work different Braak stages were included, namely with some of old AD cases classified as Braak stage II and all of young cases Braak stage V or VI. In fact, the purpose of this study was to assess the degree of neuroinflammation according to age in AD, and not an association with AD pathological severity.

Although we did not find any differences in direct comparison between EOAD and LOAD cases, there were differences in the magnitude of the neuroinflammatory markers studied when compared to the respective aged matched non-demented controls. Regarding microglial activation, there were higher microglial activation scores in EOAD when compared to aged matched controls, particularly in white matter (entorhinal and temporal regions). The simplest and most plausible explanation is that age-associated increases in microglial expression attenuate local differences in the older group [24]. However, it is worth highlighting that imaging studies of AD cases with a similar group analysis (EOAD *vs.* age matched controls and LOAD *vs.* age matched controls) have reported greater WM atrophy in EOAD than LOAD [35,36], probably reflecting a more aggressive form of the disease [35]. Recently, McAleese *et al.* [37] showed that white matter lesions differ between AD and non-demented individuals at pathological and biochemical levels, suggesting that the pathogenesis is associated with degenerative axonal changes, these probably occurring secondary to cortical AD-pathology. In our study, despite similar AD neocortical pathology severity (Braak stage V or VI) we found a greater activation of microglia in the white matter of entorhinal and temporal regions in EOAD, suggesting that age and microglia response can influence the role of AD pathology in the pathogenesis of white matter lesions. Curiously, there was a positive correlation with age at death and microglia activation in the occipital cortex in AD cases. This finding seems to contradict the regional differences reported between EOAD and LOAD, where older patients tend to have a more circumscribed disorder affecting the medial temporal lobe regions, whereas EOAD have broader neocortical involvement [17]. Functional imaging studies have shown a predilection for temporo-parietal-occipital association areas in EOAD *vs.* medial temporal cortex susceptibility in LOAD [38,39] and, recently, it was shown that with a comparable burden of fibrillar amyloid-β (as measured by Pittsburgh compound-B PET), there was greater posterior cortical hypometabolism in EOAD. This study focused on late stage AD pathology, thus probably obscuring differences that are typically reported in early disease stages [40]. However, it is interesting that microglial cell activation in the occipital cortex increases in relation to age in this AD *post mortem* sample, highlighting the complex relation between pathological findings and level of function. Serrano-Pozo [41] reported no differences in age at death in microglial cell activation in the temporal cortex of AD patients, but did not analyse other regions.

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We did not found a correlation with age and microglial scores in the control group. Certainly, complementary morphometric studies or studies directed towards functional microglial analysis would be of importance in order to interpret the current findings.

While the role of microglia in the neuroinflammatory response in AD is well established [34], several studies indicate that astrocyte-mediated inflammatory process also contribute to neurodegeneration in AD [42]. Enhanced expression of GFAP and astrocyte hypertrophy have been identified in *post mortem* tissue from AD cases [27,43,44]. As expected, we found a higher astrocyte response in AD cases when compared to aged matched control cases in the majority of the regions studied. Similar to the microglial findings in this study, the pattern of response did not differ between EOAD to LOAD. In astrocyte response, at least in the temporal region, there are no significant age-associated changes in GFAP astrocyte expression to attenuate the differences in astrocyte response pattern in AD. There is limited knowledge about ageing of astroglia and data available are controversial. In human *post mortem* tissue analysis there was no significant changes in astroglial cell counts between old and young adult brains [45,46]. In old rodents, an increase, a decrease and no change in the number of GFAP positive astroglial cells have been reported [47]. Interestingly, in the CA2/3 area of the hippocampal formation, we found significant differences in the LOAD group when compared to control groups. It is worth highlighting that the CA2/3 region is generally considered more resistant to AD pathology, leading us to speculate that the astrocyte reaction may be linked to other underlying mechanisms, which reinforces the importance of possible variations in pathology patterns that are associated with increasing age. Regarding astrocytes, age-dependent changes in morphology were also reported in rodents, with apparent significant increase volumes in neocortex (primary and secondary somatosensory cortex) and hippocampus (astrocytes from CA3 stratum molecular region) [48]. These findings were also reported to be region-specific, with GFAP-positive astroglial profiles increased in the CA1 region and lower in the entorhinal cortex [49]. In transgenic AD animals studies, astrocyte atrophy in both hippocampus (CA1 and DG) [50] and entorhinal cortex was reported [51]. While astroglial atrophy appears as a generalized process, the astrocytes that surround plaques were hypertrophic with increased surface and volume of GFAP-immunostained profiles [50]. All these findings must be interpreted with caution, taken into account the probable region-specific morphological changes associated with age and, particularly in AD mouse models, several pathological features of AD pathology are missing. Surprisingly, we did not find any differences between AD and controls in the astroglial profiles (surface, volume and soma perimeter and area) despite the higher astroglial scores found in the subiculum. The subiculum is the major output structure of the hippocampus [52] and is severely affected by AD pathology. We expected to find differences in the morphological analysis between AD and control cases in this region but the analysis did not prove it. There was also no correlation with age at death and any of the morphological parameters studied in the control group. To the best of our knowledge, there are no human tissue studies in ageing or AD that have addressed this issue previously. Some data support the concept that reactive astrocytes show hypertrophy of their intermediate filament-rich main cellular processes but do not extend to occupy a greater volume of tissue than nonreactive astrocytes [53,54]. It is also possible that, similar as reported in animal models [49], there are region-specific astroglial changes in human ageing and AD. Additionally, the analysis of astrocyte morphology in relation to proximity of AD pathology (Aβ and tau) could also be informative [50]. Nevertheless, our (negative) findings remind us that we need to translate carefully the findings of AD animal models to human tissue analysis.

This work has methodological limitations. We used a semi-quantitative scale for the assessment of the pathology, which has limitations when compared to stereology-based quantitative methods. However, we achieved a strong correlation between unbiased stereological counting performed in two regions and the semi-quantitative grading. Furthermore, there is recent data suggesting that the increase in glia in the AD brain is due to a phenotypic change in existing resting glial cells and not due to glial cell proliferation *per se* [41]. The assessment of microglia by present methods allowed us to quantify microglia activation rather than total microglia immunohistochemistry intensity 'signal'. Further studies using different methods are needed to replicate and extend these findings, and additional inflammatory markers can be added in order to better understand inflammatory process associated with AD pathology.

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Additionally, the understanding of neuroinflammation in AD warrants further study of the consequences of alterations in microglial cell and astrocyte morphology with respect to phenotype and function [34]. Finally, taken into account the higher risk of developing AD [55] and potential stronger inflammatory dysregulation in woman [56], it would be important to explore gender differences in AD associated inflammation and ageing.

Conclusion

Understanding the delicate balance between age of onset and AD pathophysiology will be important to understand the effect of interventions in dementia. Taking into account the cumulative data regarding neuroinflammatory changes associated with ageing, these differences must be addressed when modifying agents that act on the neuroinflammatory system are tested [17]. In the present study, we have shown that, overall, the neuroinflammatory pathological markers in late stage AD human tissue have a similar pattern at different ages. However, when compared to aged matched controls, the magnitude of the pathological markers in the younger AD group becomes more evident. The association between the pathological features of AD and dementia is stronger in younger old persons (75 years) than in older old persons (95 years), suggesting that additional factors are involved in the clinical expression of dementia in the oldest old [57].

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Author contributions

R.T. provided study design, did all the microscopical assessments, data analysis and wrote the paper. V.F. did the morphological astrocytes reconstructions. P.B. did all the immunohistochemistry studies. A.R. prepared sections for staining and immunohistochemistry. I.R. prepared sections for staining and immunohistochemistry. F.M. helped with discussions and paper writing. D.M.M. helped with discussions and paper writing. M.M.P. helped with discussions and paper writing. N.S. provided supervision of the study design, helped with discussions and paper writing.

Conflict of interest

The authors do not report any conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Representative examples of CD68 and Iba1 immunostaining in Alzheimer's disease (AD) and control cases in entorhinal cortex.
Figure S2. Correlation of microglia scores between CD68 and Iba1 in entorhinal and temporal regions.
Figure S3. Correlation between semi-quantitative scores and stereological counting for microglia (**A** and **B**) and astrocytosis (**C** and **D**) in subiculum and entorhinal cortex.
Table S1. Clinical/demographic characteristics, microglial and astrogliosis scores.

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3.2. Clinical study

For the study of the inflammatory profile of the CSF and serum, patients were recruited from the dementia outpatient clinic of the Department of Neurology of the Hospital Santo António – Centro Hospitalar do Porto (CHP) between February 2013 and April 2017. Patients with subjective memory complaints that underwent the study protocol were included as controls. Details of the clinical characterization of the cohort are presented here.

3.2.1. Clinical assessment and neuroimaging analysis

All patients underwent clinical assessment by a neurologist from the department every 6 months that included clinical history and neurological examination. In the first visit, detailed family history was assessed. The clinical history followed a structured proforma, which documents systematically alterations in cognition and behaviour.

The neuropsychological assessment performed in the Neuropsychology Unit of CHP included an extended battery of tests, most with normative data for the Portuguese population available (Mini Mental State Examination, Dementia Rating Scale, Auditory Verbal Learning Task, Benton Visual Retention Test, Complex Rey Figure, Digit Span, Judgment of Line Orientation, Trail Making Test, Wisconsin Card Sorting Test, Verbal Fluency and Boston Naming Test, Hospital Anxiety and Depression Scale and Neuropsychiatric Inventory).

MRI acquisition was performed at CHP, using a Philips Achieva 3.0T TX MRI scanner, and the sequence analysis included: 3D FLAIR, 3D T1 SPGR, Axial T2* (gradiente-eco), Axial T2, Coronal T2 and diffusion tensor imaging (DTI). The brain MRI acquisitions were validated by a neuroradiologist and visual scales were used (Fazekas et al., 1987; Koedam et al., 2011; Scheltens et al., 1992) to establish the clinical diagnosis.

Volumetric analysis

Data preprocessing was performed using Freesurfer (http://surfer.nmr.mgh.harvard.edu/), a tool developed for automatic brain segmentation whose technical details are

described in several publications (Dale et al., 1999; Reuter et al., 2012). Freesurfer offers consistency in its automated processing and validation studies have demonstrated that its measurements are comparable to those derived from manual tracing of brain regions (Fischl et al., 2002; Tae et al., 2008). Freesurfer has also been shown to be a highly reliable method for automated cortical thickness measurements across scanner strength and pulse sequence in all regions of the brain, with minor variability being attributed to cytoarchitectural differences of certain ROIs and difficulties with surface reconstructions in temporal lobe regions (Fjell et al., 2009).

While allowing for a full automated processing of the data, the guidelines of the tool (https://surfer.nmr.mgh.harvard.edu/fswiki/RecommendedReconstruction) suggest that the user does several corrections and validations. Three main steps were addressed: the validation of the registration, where the tkregister2 tool was used to verify the overlap of the reference brain and the subject brain using the shape of the corpus callosum, the medial line, the sulcus of the brain and the overall shape of the brain as references. Whenever necessary the tool allowed for alterations on the registration with 12 degrees of freedom; next the tkmedit tool was used for the validation of the skull extraction and the pial line, where it was verified that the latter restricted only brain tissue and did not overlap with outside structures; finally tkmedit was used to validate the segmentation of the white matter using the white matter mask and possible misclassified white matter hypointensity areas were reclassified as such.

3.2.2. Clinical cohort

Ninety-three subjects were initially screened and sixty-one subjects satisfied diagnostic criteria for inclusion in the study: 7 mild cognitive impairment (MCI-AD), 33 AD [22 early onset AD (EOAD) and 11 late onset AD (LOAD)] and 21 FTD. Nine cases with subjective memory complaints and four cases with pseudodementia/depression that underwent the study protocol were included as controls (clinical, imaging, CSF and serum studies). Four additional cases from the Minho integrative neurosciences database (MIND) biobank were used as non-inflammatory neurological controls. The following patients were excluded: 4 cases of MCI with CSF biomarkers not supporting an Alzheimer´s disease pathophysiology, 5 cases with dementia with clinical phenotype suggestive of

AD but without CSF biomarkers suggestive of this pathophysiology, 5 with psychiatric disorder and other causes, including one case with a post-mortem diagnosis of argyrophilic grain disease. One case was excluded due to elevated PCR on the day of the LP (respiratory infection). One AD case had pathological confirmation of AD with neocortical Lewy bodies. Three cases of the FTD group had a genetic form of FTD (two with a progranulin mutation and one with C9orf72 hexanucleotide repeat expansion). Two sporadic FTD cases developed motor neuron disease.

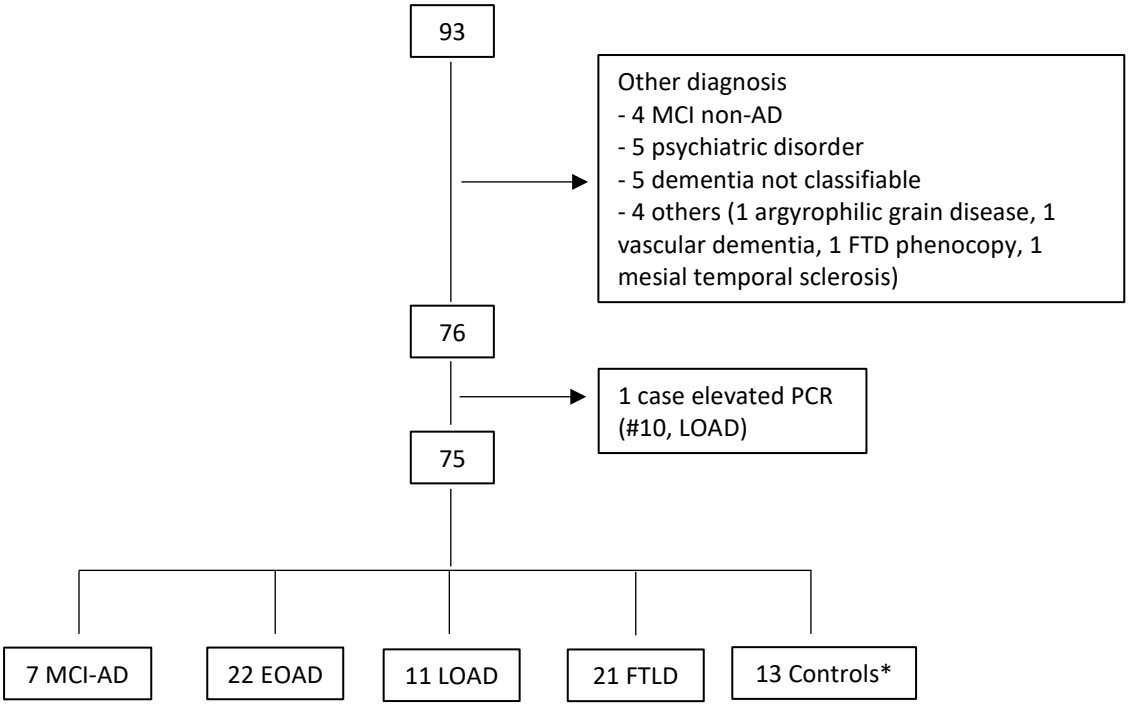

Figure 2: Clinical enrolment. *Plus 4 additional cases from MIND biobank.

The demographic, clinical and CSF biomarkers characteristics are summarized in table 3 and 4. There was an overrepresentation of male cases in the FTD group. The AD and FTD groups did not differ in global cognitive scales (MMSE and DRS-2), but the sub-scores showed that the AD group had greater impairment in memory and a trend for impairment in visuoconstruction ($p = 0.005$ and $p = 0.060$, respectively; Mann-Whitney T test).

Table 3: Demographic, clinical and CSF biomarkers characteristics

	Controls *n* = 17	**MCI-AD** *n* = 7	**AD** *n* = 33	**FTD** *n* = 21	
Sex (M:F)	6 : 11	3 : 4	13 : 20	17:4	
Age	60.4 ± 7.9	68.7 ± 6.3	62.8 ± 8.0	64.2 ± 7.5	$p = 0.068$
Education (years)	6.4 ± 3.5	6.1 ± 3.7	6.5 ± 4.4	4.9 ± 1.9	$p = 808$
MMSE	28.1 ± 1.9	25.9 ± 2.8	18.3 ± 6.5[a,b]	22.9 ± 4.3[c]	$P < 0.001$
DRS-2 (z score)	- 1.1 ± 1.6	- 1.2 ± 1.3	- 5.4 ± 2.8[a,d]	- 5.1 ± 4.1[b,e]	$p < 0.001$
CSF pTau	50.3 ± 13.1	82.0 ± 21.8[g]	88.7 ± 29.8[e,f]	39.2. ± 17.8	$p < 0.001$
CSF hTau	233.3 ± 64.3	532.3 ± 183.9[c,h]	673.3 ± 328.3[a,f]	275.4 ± 203.6	$p < 0.001$
Aβ1-42	1225.9 ± 222.7	545.9 ± 71.9[a,h]	538.4 ± 103.3[a,f]	897.0 ± 252.1	$p < 0.001$

Data are presented as means ± SD. The *p*-values in the right column refers to differences between all groups using the Kruskal-Wallis test and post-hoc testing using Dunn-Bonferroni.

[a] $p < 0.001$ vs controls

[b] $p < 0.05$ vs MCI-AD

[c] $p < 0.05$ vs controls

[d] $p < 0.01$ vs MCI-AD

[e] $p < 0.01$ vs controls

[f] $p < 0.001$ vs FTD

[g] $p < 0.01$ vs FTD

[h] $p < 0.05$ vs FTD

Table 4. Family history of dementia within the cohort.

	CONT	**MCI-AD**	**AD**	**FTD**
Autosomal Dominant	-	-	-	2
≥ 3 with dementia history	-	-	-	1
≥ 1 family member with < 65 years	-	-	3	1
≥ 1 family member with > 65 years	-	1	10	4
No family history	13	6	20	13
Total	13	7	33	21

Detailed clinical information from neurological assessment are showed in table 5. The AD and FTD group differed statistically in memory, praxis, apathy, depression symptoms, behaviour change and insight loss. Despite the small sample size, it was interesting to demonstrate such a different clinical pattern in a semi-structured clinical interview. The DRS-2 subscales showed statistically significant more severe memory deficits in the AD compared to FTD (table 6).

During the follow-up period of the study, 4 (57%) of the MCI cases converted to AD (time to conversion in years: 1.5 ± 0.6). The median of the follow-up period of the MCI group was 39 months (minimum of 25 months and maximum of 45 months).

Table 5. Detailed clinical information

	Controls *n*=13	MCI-AD *n*=7	AD *n*=33	FTD *n*=21
Clinical interview				
Memory	13/13 (100%)	8/8 (100%)	32/32 (100%)***	14/21 (66.7%)***
Language	9/13 (69.2%)	2/7 (28.6%)	22/32 (68.7%)	11/20 (55%)
Calculus	1/13 (7.7%)	1/6 (31.5%)	16/27 (59.2%)	6/17 (35.3%)
Visuo-spatial	1/13 (7.7%)	2/7 (28.6%)	18/32 (56.2%)	7/21 (33.3%)
Praxis	0/13 (0%)	0/7 (0%)	8/32 (25)*	0/21 (0%)*
Executive	8/13 (61.5%)	2/7 (28.6%)	20/32 (62.5%)	13/19 (68.4%)
Insight loss	0/13 (0%)	0/7 (0%)	12/30 (40%)**	17/20 (85%)**
Depressive symptoms	9/13 (69.2%)	2/7 (28.6%)	13/32 (40.6%)*	2/21 (9.5%)*
Apathy	1/13 (7.7%)	1/6 (31.5%)	7/32 (21.9%)*	12/21 (57.1%)*
Behavior change	0/13 (0%)	1/6 (31.5%)	3/32 (9.4%)***	20/21 (95.2%)***
Psychotic symptoms	0/13 (0%)	0/7 (0%)	3/32 (9.4%)	6/21 (28.6%)
Neurological examination				
Limb apraxia	0/13 (0%)	0/7 (0%)	4/32 (12.5%)	0/21 (0%)
Orobucofacial apraxia	0/13 (0%)	0/7 (0%)	0/32 (0%)	0/21 (0%)
Copy hands posture	0/13 (0%)	0/7 (0%)	7/32 (21.9%)	1/21 (4.8%)
Primitive reflexes	0/13 (0%)	0/7 (0%)	3/32 (9.4%)	5/21 (23.8%)
Oculomotor movem.	0/13 (0%)	0/7 (0%)	0/32 (0%)	0/21 (0%)
Bulbar signs	0/13 (0%)	0/7 (0%)	0/32 (0%)	1/21 (4.8%)
Cortical sensory loss	0/13 (0%)	0/7 (0%)	0/32 (0%)	0/21 (0%)
Pyramidal signs	0/13 (0%)	0/7 (0%)	1/32 (3.1%)	2/21 (9.5%)
LMN	0/13 (0%)	0/7 (0%)	0/32 (0%)	2/21 (9.5%)
Cerebellar signs	0/13 (0%)	0/7 (0%)	0/32 (0%)	0/21 (0%)
Myoclonus	0/13 (0%)	0/7 (0%)	2/32 (6.2%)	0/21 (0%)
Parkinsonism	0/9 (0%)	0/9 (0%)	3/32 (9.4%)	6/21 (28.6%)

Behaviour change considered positive if there was one symptom within the FTD spectrum.

*p < 0.05, **p < 0.01, ***p < 0.001 Chi-square (or Fisher's exact when appropriate) comparing AD and FTD groups.

Table 6. Neuropsychological characteristics in Control, AD and FTD groups.

	Controls *n* = 13	**AD** *n* = 33	**FTD** *n* = 21	
DRS-2				
Attention	- 0.88 ± 1.68	- 3.25 ± 2.63[b]	- 2.83 ± 4.46	$p < 0.01$
Initiation/Perseveration	- 0.87 ± 1.35	- 3.53 ± 1.99[b]	- 3.85 ± 2.18[b]	$p < 0.001$
Construction	- 0.56 ± 1.40	- 2.05 ± 2.51	- 1.11 ± 2.43	$p = 0.062$
Conceptualization	- 0.09 ± 0.75	- 2.05 ± 2.32[d]	- 2.69 ± 2.64[b]	$p < 0.01$
Memory	- 1.38 ± 1.43	- 5.42 ± 1.58[a,c]	- 3.78 ± 2.35[d]	$p < 0.001$
Total	- 1.11 ± 1.65	- 5.45 ± 2.77[a]	- 5.20 ± 4.00[b]	$p < 0.001$
Auditory Verbal Learning Task (AVLT)				
Total	- 0.75 ± 1.17	- 2.82 ± 1.00[a]	- 2.18 ± 1.28[d]	$p < 0.001$
30´	- 0.95 ± 1.26	- 3.12 ± 0.56[a]	- 2.46 ± 0.98[d]	$p < 0.001$
Recognition	26.41 ± 3.04	19.14 ± 5.37[a]	20.13 ± 7.34[d]	$p < 0.001$
Complex Rey Figure				
Copy	23.69 ± 5.27	11.59 ± 7.17[a]	18.35 ± 9.37	$p < 0.001$
Memory	- 0.50 ± 1.10	- 1.83 ± 0.56[b]	- 1.32 ± 1.07	$p < 0.01$
Total	- 0.14 ± 0.90	- 2.54 ± 1.32[a,c]	- 1.05 ± 1.89	$P < 0.001$
Corsi	- 0.19 ± 1.09	- 2.46 ± 1.61[a]	-1.24 ± 1.03	$p < 0.001$
Benton visual retention test	0.52 ± 1.32	-2.13 ± 0.78[b]	- 1.35 ± 1.56	$p < 0.05$
Verbal fluency				
Categorical	- 0.73 ± 1.22	- 1.78 ± 0.92[d]	- 1.84 ± 1.14	$p < 0.05$
Literal	- 0.60 ± 1.14	- 1.44 ± 0.98	- 1.71 ± 0.66[d]	$p < 0.05$
Judgment of line orientation	- 0.49 ± 1.61	- 2.34 ± 1.62[d]	- 1.68 ± 1.57	$p < 0.05$
Boston naming test	- 0.93 ± 3.18	- 1.33 ± 1.11[d]	- 1.31 ± 1.05	$p < 0.05$
Sentence repetition	- 0.44 ± 1.44	- 2.26 ± 2.13	- 1.32 ± 1.87	$p = 0.069$
Token	33.33 ± 5.32	24.35 ± 11.17	25.29 ± 12.51	$p = 0.240$
Digit span	-0.74 ± 1.08	- 1.39 ± 0.98	- 1.67 ± 1.03	$p = 0.081$
Trail making test A	- 0.69 ± 1.24	- 2.08 ± 1.61	- 1.40 ± 1.35	$p = 0.065$
Hospital Anxiety and Depression Scale (HADS)				
Anxiety	12.54 ± 5.49[e]	7.56 ± 4.42	8.33 ± 4.76	$p < 0.05$
Depression	9.15 ± 5.93	7.56 ± 3.36	8.40 ± 5.50	$p = 0.559$

Data are presented as means ± SD of the z scores, with exception of the recognition in AVLT, the copy of Complex Rey figure, Token and HADS that are expressed in absolute values. The *p*-values in the right column refers to differences between all groups using the Kruskal-Wallis test and post-hoc testing using Dunn-Bonferroni.

[a] $p < 0.001$ compared to controls
[b] $p < 0.01$ compared to controls
[c] $p < 0.05$ compared to FTD
[d] $p < 0.05$ compared to controls
[e] $p < 0.05$ compared to AD

Characteristics of AD according to age of onset (EOAD and LOAD)

In table 7 the demographic and clinical characteristics of the AD group are detailed according to age of onset. There were no differences in any of the characteristics showed, with exception of age.

Table 7. Demographic and clinical information of AD according to age of onset.

	Age	Sex (M:F)	Education (years)	MMSE	DRS-2 (z score)	pTau	hTau	Aβ1-42
EOAD n = 22	58.1 ± 4.7	9 : 13	7.3 ± 4.8	17.2 ± 6.7	- 5.9 ± 3.0	89.2 ± 29.3	671.8 ± 323.9	547.2 ± 90.2
LOAD n = 11	72.1 ± 3.7	4 : 7	5.2 ± 3.4	20.2 ± 5.9	- 4.6 ± 2.2	88.0 ± 32.2	676.1 ± 352.3	522.6 ± 127.1

Data are presented as means ± SD.

Table 8 and table 9 show the detailed clinical information from neurological and neuropsychological assessment respectively. EOAD cases had higher frequency of praxis and visuospatial complaints, together with more frequent signs of visuospatial impairment (copy of hand postures) and dyspraxia on neurological examination. However, these differences did not reach statistical significance ($p > 0.05$; Chi-square or Fisher exact test when applied). In neuropsychological testing EOAD group had severe impairment in attention/working memory and categorical verbal fluency when compared to LOAD group.

Table 8. Detailed clinical information in AD group according to age of onset groups.

	EOAD ***n*=22**	**LOAD** ***n*=11**
Clinical interview		
Memory	22/22 (100%)	11/11 (100%)
Language	16/21 (76.2%)	6/11 (54.5%)
Calculus	11/17 (64.7%)	5/10 (50.0%)
Visuo-spatial	14/21 (66.7%)	4/11 (36.4%)
Praxis	7/21 (33.3%)	1/11 (9.1%)
Executive	15/21 (71.4%)	5/11 (45.5%)
Insight loss	8/19 (42.1%)	4/11 (36.4%)
Depressive symptoms	9/21 (42.8%)	4/11 (36.4%)
Apathy	4/21 (19.0%)	3/11 (23.7%)
Behavior change	2/21 (9.5%)	1/11 (9.1%)
Psychotic symptoms	2/21 (9.5%)	1/11 (9.1%)
Neurological examination		
Limb apraxia	4/21 (19.0%)	0/11 (0%)
Orobucofacial apraxia	0/21 (0%)	0/11 (0%)
Copy hands posture	6/21 (28.6%)	1/11 (9.1%)
Primitive reflexes	2/21 (9.5%)	1/11 (9.1%)
Oculomotor movem.	0/21 (0%)	0/11 (0%)
Bulbar signs	0/21 (0%)	0/11 (0%)
Cortical sensory loss	0/21 (0%)	0/11 (0%)
Pyramidal signs	1/21 (4.8%)	0/11 (0%)
LMN	0/21 (0%)	0/11 (0%)
Cerebellar signs	0/21 (0%)	0/11 (0%)
Myoclonus	2/21 (9.5%)	0/11 (0%)
Parkinsonism	2/21 (9.5%)	1/11 (9.1%)

Table 9. Neuropsychological characteristics in EOAD and LOAD.

	EOAD *n* = 22	**LOAD** *n* = 11	
DRS-2			
Attention	- 3.74 ± 2.57	- 2.41 ± 2.65	*p* = 0.116
Initiation/Perseveration	- 3.97 ± 1.91	- 2.77 ± 1.98	*p* = 0.098
Construction	- 2.73 ± 2.85	- 0.88 ± 1.13	*p* = 0.106
Conceptualization	- 2.22 ± 2.59	- 1.74 ± 1.81	*p* = 0.715
Memory	- 5.45 ± 1.83	- 5.37 ± 1.11	*p* = 0.651
Total	- 5.94 ± 3.00	- 4.59 ± 2.17	*p* = 0.245
Auditory Verbal Learning Task (AVLT)			
Total	- 3.03 ± 1.04	- 2.45 ± 0.84	*p* = 0.093
30´	- 3.13 ± 0.64	- 2.88 ± 0.36	*p* = 0.052
Recognition	19.11 ± 6.43	19.20 ± 2.86	*p* = 0.866
Complex Rey Figure			
Copy	12.33 ± 8.03	10.70 ± 6.29	*p* = 0.692
Memory	- 1.98 ± 0.68	- 1.66 ± 0.34	*p* = 0.166
Total	- 2.59 ± 1.43	- 2.48 ± 1.25	*p* = 0.947
Corsi	- 3.30 ± 1.13	- 1.15 ± 1.36	*p* = 0.000**
Benton visual retention test	-1.81 ± 0.64	- 1.11 ± 1.03	*p* = 0.307
Verbal fluency			
Categorical	- 2.08 ± 0.84	- 1.27 ± 0.86	*p* = 0.030*
Literal	- 1.64 ± 1.06	- 1.11 ± 0.75	*p* = 0.085
Judgment of line orientation	- 2.82 ± 1.47	- 1.78 ± 1.70	*p* = 0.298
Boston naming test	- 1.26 ± 1.10	- 1.44 ± 1.17	*p* = 0.714
Sentence repetition	- 2.75 ± 1.90	- 1.19 ± 2.37	*p* = 0.188
Token	23.67 ± 12.12	26.00 ± 9.51	*p* = 0.752
Digit span	- 1.77 ± 0.89	- 0.80 ± 0.82	*p* = 0.013*
Trail making test A	- 2.55 ± 1.20	- 1.54 ± 1.96	*p* = 0.391
Hospital Anxiety and Depression Scale (HADS)			
Anxiety	7.12 ± 4.61	8.30 ± 4.22	*p* = 0.631
Depression	7.65 ± 3.20	7.40 ± 3.78	*p* = 0.801

Data are presented as means ± SD of the z scores, with exception of the recognition in AVLT, the copy of Complex Rey figure, Token and HADS that are expressed in absolute values. The *p*-values in the right column refers to differences between groups using Mann-Whitney T test. *$p < 0.05$; **$p < 0.001$.

Table 10 shows volumetric study performed in 17 AD (mean age ± SD, 65.35 ± 8.6; mean MMSE ± SD, 17.8 ± 7.4), 6 FTD (mean age ± SD, 58.33 ± 8.8; mean MMSE ± SD, 21.0 ± 4.5) and 6 controls (mean age ± SD, 59.50 ± 5.4; mean MMSE ± SD, 27.2 ± 2.5). The mean values are from the ratio of the structure to the total intracranial volume. The groups did not differ in terms of age at study (p = 0.214, Kruskal-Wallis test), and AD and FTD did not differ in MMSE score (p = 0.420, Mann-Whitney T test).

Table 10. Volumetric results Freesurfer analysis.

	L Hipp	R Hipp	L Cortex	R Cortex	Cortex T	L WM	R WM	WM T
AD n = 17	0.0019 ± 0.0004[a]	0.0021 ± 0.0004[a]	0.119 ± 0.017[a]	0.124 ± 0.019[a]	0.243 ± 0.036[a]	0.148 ± 0.015[b]	0.152 ± 0.014[b]	0.230 ± 0.028[b]
FTD n = 6	0.0018 ± 0.0005[a]	0.0020 ± 0.0004[b]	0.110 ± 0.021[a]	0.112 ± 0.016[a]	0.221 ± 0.037[a]	0.155 ± 0.021	0.157 ± 0.020	0.311 ± 0.041
CONT n = 6	0.0032 ± 0.0005	0.0032 ± 0.0005	0.169 ± 0.019	0.170 ± 0.019	0.338 ± 0.038	0.180 ± 0.026	0.183 ± 0.025	0.364 ± 0.050
	p < 0.01	p < 0.01	p < 0.01	p < 0.001	p < 0.001	p < 0.05	p < 0.05	p < 0.05

Results are presented as the ratio of total intra-cranial volume with means ± SD.

Legend: L- left; R – right; Hipp – hippocampus; WM – white matter; T – total. The p-values in the bottom line refers to differences between all groups using the Kruskal-Wallis test and post-hoc testing using Dunn-Bonferroni.

[a] p < 0.01 vs controls

[b] p < 0.05 vs controls

Table 11 shows the volumetric study within AD group separated according to the age at onset. There were 9 EOAD (mean age ± SD, 58.4 ± 4.8; mean MMSE ± SD, 15.0 ± 7.8) and 8 LOAD (mean age ± SD, 73.1 ± 3.7; mean MMSE ± SD, 19.7 ± 6.6). The groups did not differ in MMSE score (p = 0.247, Mann-Whitney T test).

Table 11. Volumetric results Freesurfer analysis in EOAD and LOAD.

	L Hipp	R Hipp	L Cortex	R Cortex	Cortex T	L WM	R WM	WM T
EOAD n = 9	0.0220 ± 0.0002	0.0235 ± 0.0002**	0.123 ± 0.017	0.130 ± 0.018	0.254 ± 0.034	0.152 ± 0.017	0.157 ± 0.014*	0.309 ± 0.029
LOAD n = 8	0.0174 ± 0.0004	0.0183 ± 0.0004	0.114 ± 0.018	0.117 ± 0.019	0.231 ± 0.036	0.144 ± 0.013	0.146 ± 0.012	0.290 ± 0.024

Results are presented as the ratio of total intra-cranial volume with means ± SD. *p < 0.05; **p < 0.01, Mann-Whitney T test.

3.2.3. Pro and anti-inflammatory CSF profile in young and late onset sporadic Alzheimer´s disease patients

CSF interferon γ induced chemokine (IP10) is different between young and late onset sporadic Alzheimer´s disease patients. (Manuscript in preparation)

Title

CSF interferon γ induced chemokine (IP10) is different between early and late onset sporadic Alzheimer´s disease patients

Authors and Affiliations

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Short running title: Neuroinflammatory CSF profile in young and late onset AD

Abstract

Alzheimer´s disease (AD) is a chronic neurodegenerative disorder and is the most common cause of dementia. Cumulative data suggests that neuroinflammation plays a prominent role in AD pathogenesis and there is compelling evidence, from different research groups, of age-associated dysregulation of the neuroimmune system. In this study, we sought to compare the profile of cytokines in the cerebrospinal fluid (CSF) of a group of AD patient´s according to their age and clinical characteristics. Additionally, we included another neurodegenerative dementia, frontotemporal dementia (FTD), for comparison. We found a dysregulation of pro- and anti-inflammatory cytokines in CSF of AD and FTD patients, with particular signatures for each disorder. Furthermore, in the AD group we found a positive correlation between upregulation of both pro- (IL-1β, IL-9, IL-17, G-CSF) and anti-inflammatory cytokines (IL-1ar, IL-4, IL-10, IL-13) with cognitive status at baseline, and a negative correlation with disease progression with some of them (IL-1β, IL-4, IL-17, INF- γ, FGF basic). These findings suggesting a protective role of inflammatory upregulation in early disease stages. Finally, the levels of interferon γ induced chemokine (IP-10) differed between early onset and late onset AD, suggesting an age effect on IP-10 mediated pathogenesis in AD. The study of aging as a modulating factor in the delicate balance of AD associated inflammation will be important, particularly to understand immunotherapeutic treatments currently being tested.

Introduction

Alzheimer´s disease (AD) is characterized by intracellular neurofibrillary tangles (composed by aggregates of aberrantly phosphorylated tau protein), extracellular deposits of amyloid-β (Aβ) that is accompanied by neuronal and synaptic loss, and neuroinflammation (reactive astrocytes and microglia) (Cummings and Cummings, 2004; Heneka et al., 2015; Taipa et al., 2012). Inflammation associated to AD pathology has been extensively documented and the study of proinflammatory cytokines as biomarkers for AD has gained strong interest (Wang et al., 2015). However, the data obtained from different studies are contradictory (Brosseron et al., 2014). Additionally, remains to be solved whether it is the innate immunity that enhances Aβ accumulation, thereby initiating or accelerating pathological cascades, or if the neuroinflammation is important for protection and clearance of Aβ toxic species (Taipa et al., 2016). Recent studies support the concept of a more complex interplay between innate immunity and the "proteinopathy" associated to neurodegeneration (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015). In the sporadic or late-onset AD, the complexity of AD pathogenesis is particularly evident and more distant from the simple assumption of linear causality of the original amyloid hypothesis (Scheltens et al., 2016). Regardless of clinical resemblance and neuropathological features, important differences exist between early and late onset AD (EOAD and LOAD) patients (Taipa et al., 2016). Patients with early-onset AD often present with a non-memory phenotype (Koedam et al., 2010), have a more aggressive course (Koedam et al., 2008), rarely carry the APOE ε4 allele (van der Flier et al., 2011), and have distinct patterns of early neuropathological changes (Murray et al., 2011). Our group recently showed that, in post-mortem AD brain tissue, the neuroinflammatory pathological markers share a similar pattern in EOAD and LOAD (Taipa et al., 2017). However, when compared to aged matched controls, the magnitude and extension of the pathological markers in the younger AD group was higher. Regarding the importance of neuroinflammation in the pathogenesis of AD and the differences in the neuroimmunological milieu of the aged brain, it is conceivable that the neuroinflammation associated to AD can differ between EOAD and LOAD, and contribute to or explain the clinical differences (Taipa et al., 2016).

In this study, we sought to compare the profile of cytokines and other inflammation associated protein levels in cerebrospinal fluid (CSF) of a group of AD patient´s according

to their age and clinical characteristics. Additionally, to address the issue of the possibility of a disease specific signature of neuroinflammation in AD, another neurodegenerative dementia (frontotemporal dementia) was studied for comparison.

Material and methods

Subjects

Patients were prospectively enrolled from the dementia outpatient clinic of the Department of Neurology of Centro Hospitalar do Porto (CHP) between February 2013 and April 2017. All patients underwent a standard battery of examinations, including medical and family history, physical and neurological examination, screening laboratory tests, neuropsychological evaluation (including Mini Mental State Examination, Dementia Rating Scale, Auditory Verbal Learning Task, Benton visual Retention Test, Complex Rey Figure, Digit Span, Corsi Test, Judgment of Line Orientation, Trail Making Test, Wisconsin Card Sorting Test, Verbal fluency and Boston Naming Test, Hospital Anxiety and Depression Scale and Neuropsychiatric Inventory), brain Magnetic Resonance Image (MRI) and CSF analysis. The clinical history followed a structured proforma, which included a systematic recording of changes in cognition and behaviour. Neuropsychological assessment, MRI acquisition and lumbar puncture (LP) were performed within four weeks after first clinical visit.

The diagnosis of AD were established according to the recent NIA-AA 2011 criteria (McKhann et al., 2011). The onset of symptoms had to be equal or less than four years. Patients were classified as EOAD and LOAD using the cut-off age of 65. Due to the lack of established cut-off values for AD CSF biomarkers in our laboratory, the tau/Aβ42 ratio of >0.52 was used to define a positive CSF profile for Alzheimer´s disease pathology (Duits et al., 2014). Patients with subjective memory complaints and pseudo-dementia who underwent study protocol were included as controls. A group of patients with frontotemporal dementia (FTD) was included for comparison. (Rascovsky et al., 2011). Additionally, four cases from the Minho integrative neurosciences database (MIND) biobank, with no history of inflammatory disorder or cognitive impairment, were added to the control group in the CSF studies. Patients with significant vascular brain damage were excluded (strategically placed and/or large vessel infarcts and/or white matter lesions setting a Fazekas scale > 2). Exclusion criteria additionally included the presence

of any chronic inflammatory disease, chronically use of steroidal anti-inflammatory drugs or immunosuppressive agents. Additionally, a blood sample was taken in the same day of LP and C reactive protein (CRP) was measured to exclude systemic inflammation. The study was approved by the ethical committee of CHP. All patients (or their surrogates) provided informed consent.

Blood collection and CSF collection

Blood and CSF collection was performed at CHP. Whole blood samples were allowed to sit at room temperature for a maximum of 30 minutes after collection. Separation of the clot was done by centrifugation at 3000rpm at room temperature for 15 minutes. CSF samples were obtained in polypropylene tubes by LP at the L4/L5 or L3/L4 interspace. Serum and CSF samples were aliquoted, immediately frozen and stored at –80 °C until analysis.

Aβ42, tau and p-tau determination

CSF Aβ42, phospho tau and total tau protein levels were determined using commercially available Aβ 42 (Innotest β-amyloid 1–42), t-tau (Innotest hTau-Ag), p-tau (Innotest Phospho-tau 181P) ELISA assay kits (Innogenetics, Gent, Belgium) according to the manufacturer's instructions. Blood contamination of the CSF was excluded by cytochemical analysis.

Cytokines determination

Cytokines were measured using the Bio-Plex Pro Human Cytokine 27-Plex Immunoassay kit according to instructions from the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). These include: basic fibroblast growth factor (FGF basic), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (INF-γ), interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, INF-γ inducible protein (IP-10), monocyte chemotactic protein 1 (MCP-1), macrophage Inflammatory proteins-1α (MIP-1α), MIP-1β, platelet-derived growth factor (PDGF-BB), tumor necrosis factor alfa (TNF-α) and vascular endothelial growth factor (VEGF). Plates were read in a BioPlex® MAGPIXTM Multiplex Reader (Bio-Rad Laboratories), and data was analysed using Bio-Plex Manager

MP 6.1 (Bio-Rad Laboratories). All samples were quantified in duplicate, and a coefficient of variation (CV) lower than 30% was considered for sample inclusion in analysis, for each cytokine. Moreover, for the standard curves, a 4PL or 5PL regression was used, standards with CV values higher than 30% were excluded, and all standards presented a concentration observed/expected between 70 and 130%. Concentration values below the lowest standard, or above the highest standard, extrapolated by the analysis software were also included in the statistical analysis.

Statistical analysis

Descriptive statistics, including means, standard deviations, frequencies and percentages were calculated. To compare the groups under analysis in the clinical and demographical variables, we performed a One-Way Anova and t-test for quantitative measures and the Chi-Square test specifically for gender. According with the Levene´s test for equality of variances, the Welch correction was applied if unequal variances were observed and the post-hoc test was performed accordingly (Bonferroni for equal variances assumed and Games Howell for unequal variances). For the analysis of cytokines profile, three planed contrasts analysis were performed: the first comparing dementia group (AD + FTD) with controls, the second comparing AD group with FTD group, and the third comparing EOAD with LOAD cases. Pearson correlation coefficient was calculated to assess the relation between age, cytokines and cognitive data. Data was analysed using IBM SPSS Statistics software (version 22.0) and GraphPad Prism, version 6.01. A p-value of less than 0.05 was considered statistically significant.

Results

Fifty-four patients met the diagnostic criteria of AD (n=33; 22 EOAD and 11 LOAD) or FTD (n=21). One AD case was excluded due to elevated CRP in the day of LP (respiratory infection). Nine subjects with subjective memory complaints and four cases with pseudo-dementia were included as controls.

One AD case had pathological confirmation of AD with neocortical Lewy bodies. Three cases of the FTD group had a genetic FTD form (two with a progranulin mutation and one with C9orf72 hexanucleotide repeat expansion). The main demographic and clinical characteristics of the study groups are reported in table 1.

Table 1. Demographic and clinical information.

	Controls (n = 17)	**AD (n=33)**	**FTD (n=21)**	**Statistical test result**
Sex (M:F)	6 : 11	13 : 20	17:4	$X^2(2, n=70) = 10.5$; $p < 0.01$
Age	60.4 ± 7.9	62.8 ± 8.0	64.2 ± 7.5	$F(2, 67) = 1.051$; $p = 0.355$
Education (years)	6.4 ± 3.5	6.6 ± 4.4	4.7 ± 1.8	$F(2, 61) = 1.445$; $p = 0.244$
MMSE	28.2 ± 1.9	18.3 ± 6.5[a,b]	22.9 ± 4.3[c]	$F(2, 29.8) = 32.1$; $p < 0.001$
DRS-2 (z score)	- 1.1 ± 1.6	- 5.4 ± 2.8[a]	- 5.2 ± 4.0[c]	$F(2, 60) = 9.98$; $p < 0.001$
CSF pTau	50.3 ± 13.2	88.8 ± 29.8[a,d]	39.2 ± 17.8	$F(2, 38.5) = 28.1$; $p < 0.001$
CSF hTau	233.3 ± 64.3	673.3 ± 328.4[a,d]	275.4 ± 203.6	$F(2, 36.9) = 25.1$; $p < 0.001$
CSF Aβ1-42	1225.9 ± 222.7	538.4 ± 103.4[a,d]	897.0 ± 252.1[c]	$F(2, 30.0) = 67.9$; $p < 0.001$

Legend: Values are given as the mean and standard deviations.

[a] $p < 0.001$ *vs.* controls

[b] $p < 0.05$ vs FTD

[c] $p < 0.01$ vs Controls

[d] $p < 0.001$ *vs.* FTD

There was an overrepresentation of male cases in the FTD group compared to controls and AD group.

In table 2 the main demographic and clinical characteristics of AD patients according to age of onset (i.e., EOAD vs. LOAD). are presented.

Table2. Demographic and clinical information for the EOAD and LOAD patients.

	EOAD (n=22)	**LOAD (n=11)**	**Statistical test result**
Sex (M:F)	9 : 12	4 : 7	$X^2(1, n = 33) = 0.0635; p = 0.801$
Age	58.1 ± 4.7	72.1 ± 3.7	t (31) = - 8.61; p <0.001
Education (years)	7.3 ± 4.8	5.2 ± 3.4	t (29) = 1.29; p = 0.206
MMSE	17.2 ± 6.7	20.2 ± 5.9	t (27) = -1.23; p = 0.231
DRS-2 (z score)	- 5.9 ± 3.0	- 4.6 ± 2.2	t (28) = -1.31; p = 0.201
CSF pTau	89.2 ± 29.3	88.0 ± 23.2	t (4) = - 0.745; p = 0.498
CSF hTau	671.8 ± 323.9	676.1 ± 353.3	t (4) = - 0.746; p = 0.502
Aβ1-42	547.2 ± 90.2	522.6 ± 127.1	t (4) = - 0.123; p = 0.908

Legend: values are given as the means and standard deviations.

CSF interleukins according to clinical diagnosis

As illustrated in table 3, the majority of the cytokines appeared to be more elevated in AD patients than in controls. Of interest, the FTD group appeared to be in an intermediate position.

Table 3. Mean values of inflammatory molecules in the CSF of patients and controls (z scores).

	Controls	AD	FTD		EOAD	LOAD
IL-1β	- 0.32 ± 0.95	0.23 ± 0.87	0.13 ± 1.02		0.18 ± 0.92	0.34 ± 0.81
IL-1ar	- 0.55 ± 0.76	0.44 ± 0.98	0.08 ± 0.95		0.35 ± 0.94	0.63 ± 1.08
IL-2	- 0.32 ± 0.98	0.23 ± 0.84	0.04 ± 0.95		0.18 ± 0.94	0.33 ± 0.62
IL-4	- 0.42 ± 0.83	0.28 ± 0.90	0.06 ± 0.84		0.11 ± 0.87	0.62 ± 0.90
IL-5	- 0,21 ± 1.05	0.35 ± 0.82	0.26 ± 0.99		0.31 ± 0.84	0.44 ± 0.81
IL-6	- 0.31 ± 1.00	0.10 ± 0.96	0.14 ± 0.96		0.09 ± 1.06	0.13 ± 0.76
IL-7	- 0.41 ± 0.74	0.44 ± 1.19	0.24 ± 0.80		0.30 ± 0.94	0.73 ± 1.59
IL-8	- 0.40 ± 1.07	0.26 ± 0.83	0.07 ± 1.13		0.08 ± 0.80	0.63 ± 0.79
IL-9	- 0.30 ± 0.87	0.44 ± 0.95	0.09 ± 0.92		0.26 ± 0.85	0.80 ± 1.09
IL-10	- 0.53 ± 0.88	0.38 ± 0.95	0.10 ± 0.84		0.21 ± 0.80	0.72 ± 1.16
IL-12 (p70)	- 0.24 ± 0.92	0.20 ± 0.92	0.18 ± 1.00		0.14 ± 0.96	0.30 ± 0.85
IL-13	0.01 ± 0.72	0.40 ± 1.07	- 0.01 ± 0.83		0.20 ± 0.90	0.85 ± 1.33
IL-15	- 0.53 ± 0.86	0.34 ± 1.13	- 0.20 ± 0.84		0.11 ± 1.00	0.81 ± 1.26
IL-17	- 0.25 ± 0.93	0.22 ± 0.95	0.02 ± 1.02		0.21 ± 1.09	0.25 ± 0.63
INF-γ	- 0.29 ± 0.80	0.18 ± 0.83	0.07 ± 0.88		0.02 ± 0.88	0.70 ± 0.84
IP-10 (CXCL10)	0.24 ± 1.13	0.17 ± 0.68	- 0.18 ± 0.63		- 0.21 ± 0.52	0.43 ± 0.76
Eotaxin	- 0.30 ± 0.74	0.40 ± 1.03	0.23 ± 1.04		0.21 ± 1.02	0.79 ± 0.98
FGF basic	- 0.25 ± 0.88	0.23 ± 0.94	- 0.02 ± 1.12		0.24 ± 1.04	0.21 ± 0.76
G-CSF	- 0.52 ± 0.61	0.45 ± 1.11	0.00 ± 0.82		0.33 ± 1.00	0.70 ± 1.33
GM-CSF	- 0.29 ± 0.98	0.28 ± 0.94	-0.08 ± 0.90		0.07 ± 0.93	0.70 ± 0.84
MCP-1 (CCL2)	0.11 ± 1.02	- 0.35 ± 0.88	0.41 ± 1.12		- 0.44 ± 0.94	- 0.18 ± 0.78
MIP-1α (CCL3)	- 0.27 ± 0.87	0.23 ± 0.91	0.36 ± 1.21		0.16 ± 0.79	0.39 ± 1.14
MIP-1β (CCL4)	- 0.31 ± 0.74	0.12 ± 0.89	0.14 ± 1.30		-0.11 ± 0.65	0.60 ± 1.14
PDGF-BB	- 0.36 ± 0.77	0.40 ± 1.04	0.23 ± 0.90		0.38 ± 1.02	0.45 ± 1.14
RANTES (CCL5)	- 0.12 ± 0.45	0.19 ± 1.44	- 0.19 ± 0.05		- 0.08 ± 0.58	0.69 ± 2.32
TNF- α	- 0.41 ± 1.00	0.33 ± 1.00	0.11 ± 0.95		0.33 ± 1.00	0.34 ± 1.04
VEGF	0.40 ± 0.97	0.07 ± 1.05	- 0.35 ± 0.75		0.11 ± 1.14	0.02 ± 0.92

Legend: values are given as the means and standard deviations

Abbreviations: CCL – C-C motif chemokine ligand; CXCL – C-X-C motif chemokine ligand

Differences between groups

In order to test the differences in the CSF inflammatory profile of the different groups, contrast analysis was applied with the following steps: (1) controls vs dementia, (2) FTD vs AD and (3) EOAD vs LOAD.

1. Controls vs dementia

The CSF of the "dementia" group had significant higher values of IL-1ra, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-15, eotaxin, G-CSF, GM-CSF, MIP-1α (CCL3), PDGF-BB and TNF-α compared to controls (Figure 1).

2. FTD vs AD

When comparing the FTD and AD patients we found that the IL-15 levels were higher in AD group and, inversely, MCP-1 levels were higher in FTD group (Figure 2).

3. EOAD vs LOAD

Of interest, within the AD group, when comparing the EOAD and the LOAD patients we observed that the IP-10 were lower in EOAD when compared to the LOAD group (Figure 3).

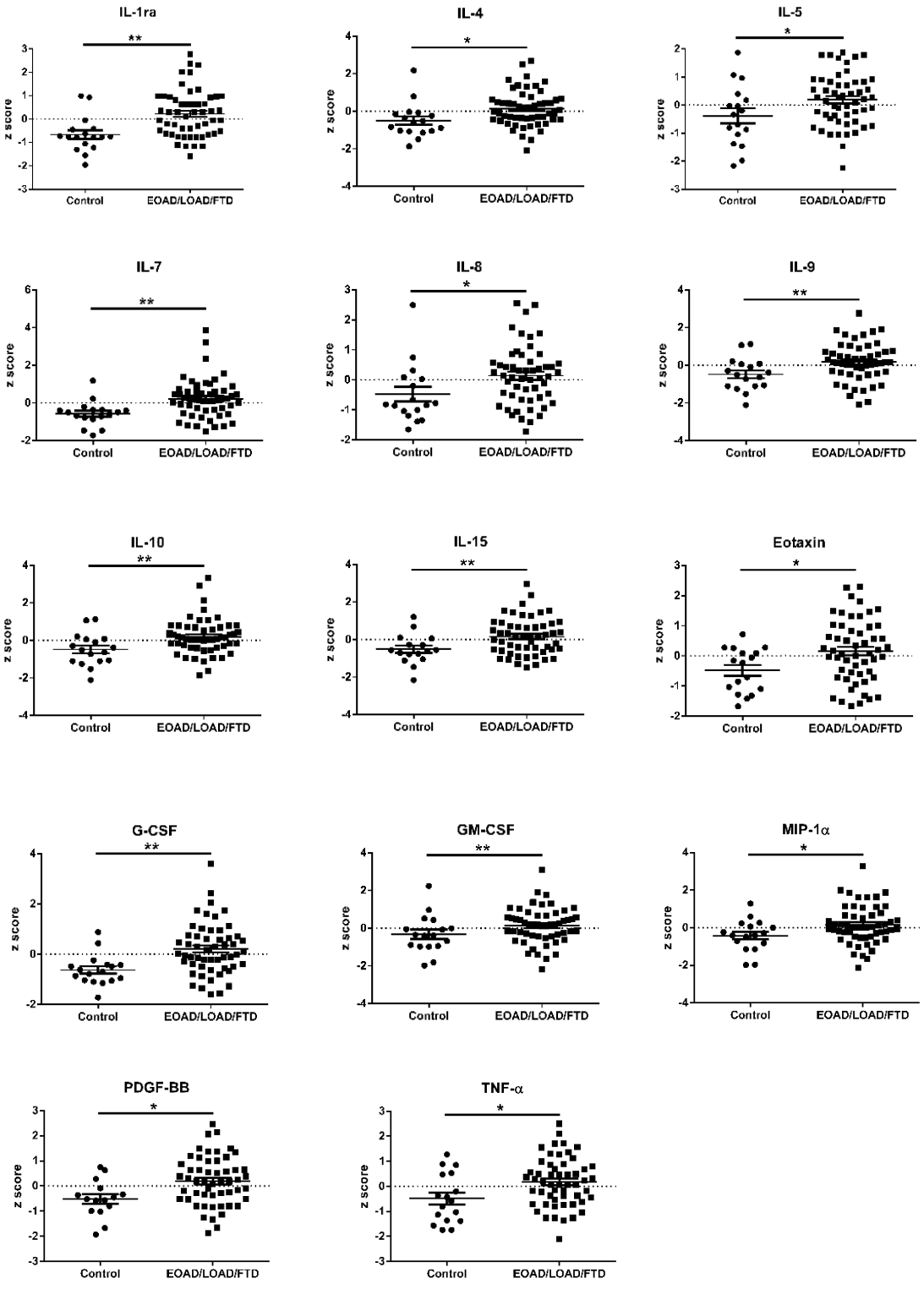

Figure 1. Differences between dementia cases (AD and FTD) and control groups. Values of cytokines are presented in z scores, contrast analysis. *p < 0.05; **p < 0.01

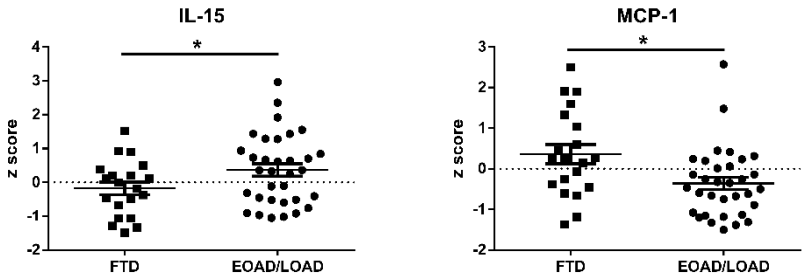

Figure 2. Differences between AD and FTD cases. Values of cytokines in z scores, contrast analysis. *p < 0.05.

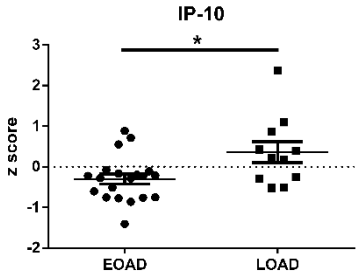

Figure 3. Differences between EOAD and LOAD. Values of cytokines in z scores, contrast analysis. *p < 0.05.

Correlation to age

In AD group there was positive correlation with IP-10 and MIP-1β (Figure 4a and b). There was a trend for positive correlation with MCP-1 ($r = 0.340$, $p = 0.053$). In FTD group there was a positive correlation with MCP-1 (Figure 4c). No correlation was found in control group.

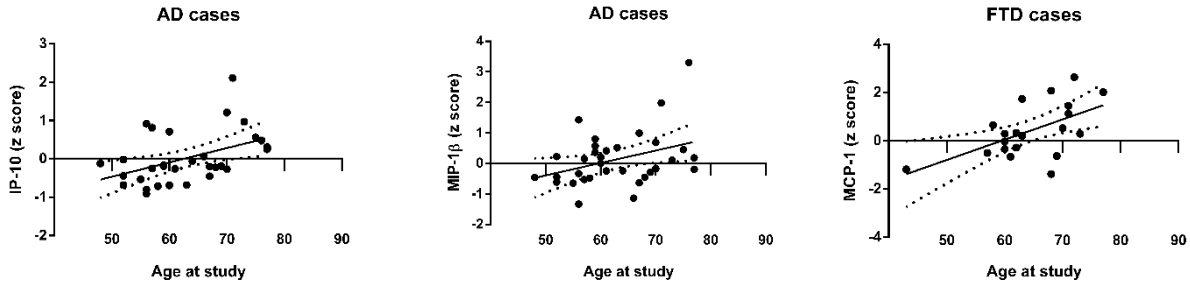

Figure 4. Correlation between age and cytokines in AD (IP-10 and MIP-1β) and FTD (MCP-1). a) $r = 0.449$, $p = 0.011$; b) $r = 0.365$, $p = 0.037$; c) $r = 0.569$, $p = 0.009$.

Correlation between cytokines and cognitive characteristics and disease progression

In the AD group there were several inflammatory molecules that correlated positively with cognitive status (DRS-2) and negatively with cognitive decline (DRS-2 score decrease after 1 year) controlling for the DRS-2 score at first visit (table 4).

In the FTD group there was no significant correlation between any of cytokines and cognitive status at baseline. IL-7 correlated negatively with DRS-2 in the second evaluation at 12 months ($r = -0.571$; $p < 0.05$) and positively with disease progression ($r = 0.730$; $p < 0.01$), both controlling for DRS-2 score at the first visit.

In the control group there was no correlation between any of the molecules and cognitive status at baseline.

Table 4. Correlation between cytokine levels and cognitive status (DRS-2), and cytokine levels and cognitive decline at 1 year.

	DRS-2 (T1)	DRS-2 (T2)	DRS-2 (T1 - T2)
IL1β	**0,366***	**0,443***	**-0,427***
IL1ar	**0,367***	0,108	-0,060
IL2	0,167	0,286	-0,363
IL4	**0,364***	0,377	**-0,419***
IL5	0,301	0,358	-0,283
IL6	0,325	**0,455***	-0,394
IL7	0,354	0,205	-0,221
IL8	-0,051	-0,007	-0,155
IL9	**0,406***	0,386	-0,359
IL10	**0,446***	0,353	-0,288
IL12	0,305	0,357	-0,303
IL13	**0,393***	0,233	-0,120
IL15	0,360	0,211	-0,246
IL17	**0,384***	0,407	**-0,433***
Eotaxin	0,339	**0,473***	-0,260
FGF basic	0,258	**0,546****	**-0,533****
G-CSF	**0,369***	0,342	-0,342
GM-CSF	0,197	0,297	-0,410
INF-γ	0,233	0,400	**-0,446***
IP10	0,036	0,033	0,111
MCP-1	0,201	0,000	0,015
MIP-1α	0,244	0,127	-0,056
PDGF-BB	0,356	0,301	-0,276
MIP-1β	0,344	0,322	-0,282
RANTES	0,060	0,168	-0,256
TNF-α	0,279	0,222	-0,231
VEGF	0,166	0,177	-0,175

Legend: T1 – first neuropsychological evaluation; T2 – second neuropsychological evalulation (12 months later). Columns for DRS-2 T2 and DRS-2 (T1 – T2) represent partial correlations controlled for DRS-2 T1. * $p < 0.05$; ** $p < 0.01$ (Pearson correlation).

Discussion

This study supports the concept that inflammatory dysregulation plays a role in the pathogenesis of both neurodegenerative dementias, AD and FTD. Furthermore, the inflammatory CSF profile displays a specific signature for each disorder, and age can be associated to neuroinflammatory particularities in EOAD and LOAD. Despite conflicting data in the literature, our results support the existence of an upregulation of pro- and anti-inflammatory cytokines in the CSF of AD patients (Brosseron et al., 2014). Recently, two experiments (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015) demonstrated that an anti-inflammatory cytokine (IL-10) inhibits Aβ clearance by microglia, worsening cognitive decline in mouse models of AD. These results are in line with the perception that inflammation in the context of AD is not exclusively detrimental or beneficial, but has to be fine-tuned (Michaud and Rivest, 2015). Moreover, the inflammatory changes seem to be time dependent in the disease progression (Galimberti et al., 2006). In our study, we found that cognitive status in AD correlated positively with a broad range of pro- and anti-inflammatory molecules, supporting the notion that changes of inflammatory profile in the CSF is an early event (Brosseron et al., 2014). Interestingly, we found a negative correlation with disease progression at 12 months with some of the cytokines that correlated positively with baseline cognitive status. Contrary to our findings, some studies reported faster disease progression in association with higher levels of some inflammatory cytokines, namely eotaxin (CCL2) in prodromal AD (Westin et al., 2012). However, an in vivo imaging study has recently suggested that there may be two peaks of microglia activation in the AD disease trajectory, an early protective peak and a later pro-inflammatory peak (Fan et al., 2017). Moreover, another similar methodological approach showed that microglia activation was positively correlated with MMSE scores in AD patients, and that AD patients with slow decline had higher translocater protein-binding (considered a marker of microglial activation) (Hamelin et al., 2016). These findings are in agreement with our results, strengthening the concept of a dynamic process that can have different consequences (protection vs injury) depending on the time and stage of disease. This relation need further studies, analysing the pattern of the different inflammatory markers in different time points of the disease. When contrast analysis was further applied within dementia groups, MCP-1 and IL-15 levels were found to be different between AD and FTD groups. CSF levels of MCP-1 have

been previously reported to be significantly increased in FTD compared to age matched controls (Galimberti et al., 2006). More recently, this finding was associated specifically to sporadic FTLD and not in progranulin (GRN) mutation carriers (Galimberti et al., 2015). Comparing with other studies, our FTD CSF study cohort is relatively large with only three cases with familial forms of FTLD (2 with GRN mutation). The literature has provided inconsistent results regarding CSF MCP-1 levels in AD, with some studies reporting an upregulation in AD and MCI patients (Brosseron et al., 2014; D. Galimberti et al., 2006) and others unchanged levels (Brosseron et al., 2014; Johansson et al., 2017; Kauwe et al., 2014). Similarly to Galimberti et al (2015), we found that MCP-1 levels increase with age in FTD (Galimberti et al., 2015) and there was a similar trend in the AD group. We did not find such correlation in controls, but a larger sample will be important to understand the possible role of this cytokine in normal or pathological aging. Furthermore, longitudinal data from AD patients and animal models will be important in our understanding of the roles of MCP-1 in these disorders. Regarding IL-15, a small study found increased levels in the CSF of patients with AD and FTD (Rentzos et al., 2006). More recently, Galimberti et al. (2015) found increase IL-15 CSF levels only in GRN mutation carriers (Galimberti et al., 2015), but not in sporadic FTD. In the first study, only 7 FTD cases were analysed. Similarly to our study, IL-15 was not correlated with age, MMSE or disease progression in AD or FTD (Rentzos et al., 2006). Our study does not suggest a common role for MCP-1 and IL-15 in neurodegeneration in both conditions, at least in this stage of the disease. The first is apparently associated to (sporadic) FTD and the later to AD pathogenesis.

When we further analysed AD according to age of onset, we found that IP-10 levels are decreased EOAD compared to LOAD. Higher IP-10 levels have been reported in AD patients, particularly in mild AD (Galimberti et al., 2006). In that study, AD patients were older at onset, had longer disease duration and lower MMSE scores than in the current study. No CSF AD biomarkers were available at that time, potentially decreasing diagnostic certainty. Despite the fact that no statistical differences were found in cognitive assessment at baseline of both groups (EOAD and LOAD), we stratified both groups in a similar way according to MMSE score (< or > 15) and we did not find differences in IP-10 levels (data not shown). Our results raises the possibility of an age effect in IP-10 dysregulation in AD pathogenesis. IP-10 has been shown to be

upregulated in reactive astrocytes of AD brains and frequently associated to amyloid plaques (Xia et al., 2000). Interestingly, the same study showed that IP-10 is up regulated in a co-ordinated manner with another chemokine, MIP-1β. The association between the pathological features of AD and dementia is stronger in younger than in older persons, suggesting that additional factors are involved in the clinical expression of dementia in the older patients (Savva et al., 2009).

Our study has some limitations, namely, the small sample size and the control group [non-inflammatory neurological controls (Teunissen et al., 2013) and not true "healthy non-demented" controls]. However, the patients have robust clinical diagnoses and long follow-up periods. Serum analysis would also be important to mirror systemic inflammatory activity, particularly taking into account the recent view of a frequent communication of the immune activities between periphery and the central nervous system (Wang et al., 2017).

In conclusion, this study supports a pro and anti-inflammatory immune dysregulation in AD and FTD, together with findings of particular signatures for each disorder (IL-15 for AD and MCP-1 for FTD). Furthermore, it supports a possible protective role of inflammatory upregulation in early disease stages and suggests an age effect on IP-10 mediated pathogenesis in AD. These results must be interpreted in this particular disease stage. A longitudinal view (from prodromal to late stage AD) in EOAD and LOAD will be of paramount importance to understand the role of inflammation in disease pathogenesis and developing treatment strategies that target this mechanism.

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4. Discussion and conclusions

Alzheimer´s disease (AD) was first identified more than 100 years ago (Alzheimer, 1906), but it took more than 70 years to be recognized as the most common cause of dementia (Katzman, 1976). Afterwards, it became a major area of research and is now one of the great health-care challenges of the 21st century. Typically, AD patients present with memory impairment and executive dysfunction interfering with daily activities. However, a subset of patients present with language, visual, praxis, or executive problems before and more pronounced than memory deficits. Such atypical presentations are more common in early-onset AD (Scheltens et al., 2016). EOAD seldom carry the APOE ε4 allele (van der Flier et al., 2011), have more severe brain hypometabolism with similar amyloid burden (Rabinovici et al., 2010), and have greater neurofibrillary tangle burden in cortical association areas (Murray et al., 2011). Despite the small sample, the clinical characteristics of the patients included in this Thesis are in line with the literature and confirm the concept that age at onset remains important in determining the clinical phenotype (van der Flier et al., 2011). In addition, in the small subset of patients where MRI volumetric studied was completed, the LOAD patients had smaller hippocampus compared to EOAD patients. This is in agreement with previous volumetric studies that showed that younger patients have better preserved hippocampi (Frisoni et al., 2007; Karas et al., 2007). Despite the overlap of clinical and pathological features, age seems to define some specific characteristics associated to AD. Some factors such APOE genotype are now well known to play important roles in determining the clinical phenotype (van der Flier et al., 2011), but other factors related to age are yet to be discovered. In this work, taking into account the cumulative data associating inflammation to aging and neurodegenerative disorders, we aim to determine if neuroinflammation associated to AD differs between EOAD and LOAD. The work was divided in post-mortem pathological and clinical studies. In both approaches other than AD, FTD was included for comparison as a different type of neurodegenerative dementia.

Despite being beyond the scope of the main objectives of this work, it was remarkable to demonstrate marked differences in the clinical interview between the two dementia

groups studied. In particularly, the early loss of insight in the FTD group, that was included as one of five core diagnostic features in the previous FTD diagnostic criteria (Neary et al., 1998) should be highlighted. Recently, a group using a Self-Consciousness Questionnaire reported similar differences between the AD and FTD patients (Arroyo-Anlló et al., 2016). Despite being more severely affected in AD, memory was also a very common complaint at symptom onset (70%) and significantly affected in neuropsychological assessment in the FTD group. Moreover, the FTD cases also showed reduce hippocampal volumes when compared to controls. This highlights the clinical difficulties in achieving the correct diagnosis in FTD patients with severe memory deficits at presentation (Graham et al., 2005; Hornberger and Piguet, 2012). Furthermore, a recent study assessed a large pathologic series of FTD syndromes without AD, reporting β-amyloid deposition in 38% (21/56) in patients with behavioral variant FTD. In patients with progranulin mutations, despite the younger age at death, a large proportion of them (43%) demonstrated β-amyloid deposition (Tan et al., 2017). One of the cases of the cohort with definitive diagnosis of FTD due to a pathogenic progranulin variant had decrease levels of Aβ1-42 in the CSF. This case highlights the importance of being aware that AD and FTD, in addition to overlapping clinical presentations, exceptionally can also have overlapping CSF biomarkers profile.

The amyloid cascade hypothesis was proposed more than two decades ago (Hardy and Higgins, 1992) and, even though the bulk of data continuing to support a role for Aβ as the primary initiator of AD pathogenesis, multiple layers of complexity have emerged. We still do not understand the precise biological changes that cause AD, why it can present with different phenotypes, why it progresses more rapidly in some than in others, and how the disease can be prevented, slowed or stopped. In the sporadic or late-onset AD, the complexity of AD pathogenesis is even more evident and more distant from the simple assumption of linear causality by the original amyloid hypothesis (Scheltens et al., 2016). Together with extracellular deposits of Aβ and neurofibrillary tangles, reactive gliosis and neuroinflammation are hallmarks of AD. The post-mortem analysis of AD brains has provided pioneering evidence of inflammation in the brain of AD subjects but, until recently, the neuroinflammation associated with AD pathology was considered to be a secondary event to neurodegeneration (Salter and Stevens,

2017). However, recent genetic and transcriptomic studies have identified microglia-related pathways as central to AD risk and pathogenesis (Guerreiro et al., 2013; Zhang et al., 2013). In particular, polymorphisms in the genes *CD33* and *TREM2* directly link impaired microglial and macrophage phagocytosis of Aβ to increased susceptibility to AD (Zuroff et al., 2017). Complement proteins are upregulated in the brain of several AD mouse models and cumulative data implicate microglia, complement, and other immune-related pathways as early mediators of synaptic dysfunction even before the presence of plaques and overt inflammation (Salter and Stevens, 2017). Additionally, the astrocytes can be induced to shift to a what is called A1 state, pro-inflammatory and neurotoxic, and recent research showed that aberrant microglia signaling can induce this astrocyte state (Liddelow et al., 2017).

Taking into account the role of microglial driven neuroinflammation in the pathophysiology of neurodegenerative disorders, in a first post-mortem study, we were able to demonstrate that AD and FTD had relative specific patterns of microglial cell activation. There was more pronounced microglial activation in frontal subcortical white matter in FTD and more prominent involvement of temporal regions in AD (Taipa et al., 2017). This pattern probably reflects the distribution of the pathologic signature of both conditions and correlates with the *in vivo* imaging studies that showed more extensive white matter degradation in FTD than AD (Whitwell et al., 2010; Zhang et al., 2009). Detailed assessment of the hippocampal structures showed some microglial circuit specific patterns that can help explain some of the clinical overlap between AD and FTLD-TDP, namely in memory deficits. Our findings support the contribution of extra–hippocampal structures for the episodic memory deficits found in FTLD-TDP patients (Hornberger et al., 2012), namely within connection pathways, as we found a prominent microglia activation in the hippocampal white matter (i.e. alveolus) without CA1 involvement. The later affected in AD. Despite a detailed neuroanatomical picture, our findings do not clarify the temporal relationship between microglia activation and disease pathogenesis. In vivo studies with 18kDa translocator protein (TSPO), the only microglial marker available for *in vivo* imaging, demonstrated a tendency toward increase TSPO binding in AD patients (Schain and Kreisl, 2017). In FTD, increased TSPO has also been reported in a small study (Cagnin et al., 2004), but PET studies using

second-generation radioligands for TSPO are lacking (Schain and Kreisl, 2017). There are recent studies addressing this temporal dynamics in AD: while one reveals an increase in early stages (MCI with Alzheimer´s disease pathophysiology) (Parbo et al., 2017), another shows that such relationship may be non-linear (Fan et al., 2017). Given that pathological studies typically depict an end-stage picture of the process, additional studies in low Braak stages (with and without known cognitive decline) could be helpful in addressing this issue. In light of the increasing rarity of human brain tissue for study, particularly in early stages of dementia and cognitively well characterized "non-neurological" donors, multicenter studies would be helpful; this is specially true for the study of frozen tissue in addressing deeper levels of mechanistic neurodegenerative pathways.

In the second study of this Thesis, we characterized the pattern of microglial activation and astrogliosis in multiple anatomical areas in clinically and pathologically confirmed AD and non-demented control cases in relation to age. There is strong support for the role of Aβ accumulation in familial AD and sporadic (typically late onset) AD (Musiek and Holtzman, 2015). However, sporadic late-onset AD is a more complex disorder and the linear model of Aβ toxicity is very likely to be incorrect. Sporadic late-onset AD accounts for more than 99% of all cases (Campion et al., 1999), and the ratio of LOAD patients to all AD patients continues to increase because aging is a primary risk factor aligned with aging of the world population. Brain aging is a dynamic process, with clinical and experimental evidence for a shift for pro-inflammatory status (Norden and Godbout, 2013), together with an imprecisely defined process of "immunosenescence" (Di Benedetto et al., 2017). With this study, we wanted to know if AD associated inflammation pathology markers differ between sporadic AD patients according to their age at onset (EOAD or LOAD). In fact, our results showed that overall the pattern of neuroinflammation (microglia activation and astrogliosis) is similar between both age AD groups. It is important to highlight that our study focused on late stage AD pathology, thus probably obscuring differences that are typically reported in early disease stages (Ossenkoppele et al., 2015a). Nevertheless, when compared to age matched controls, there were higher microglial activation scores in EOAD, particularly in temporal white matter. The simplest and most plausible explanation for this finding is that age-

associated increases in microglial expression attenuate local differences in the older group. However, it is worth noting that imaging studies have reported greater white matter atrophy in EOAD than LOAD (Canu et al., 2012; Migliaccio et al., 2012) and recently, a pathological study suggested that the pathogenesis of the white matter lesions in AD are secondary to cortical AD-pathology (McAleese et al., 2017). In our study, despite similar AD neocortical pathology severity (Braak stage V or VI) we found a greater activation of microglia in temporal white matter regions in EOAD, suggesting that age and microglia response can influence the role of AD pathology in the pathogenesis of white matter lesions. There is evidence linking senescent astrocytes to an increase risk of sporadic AD (Bhat et al., 2012). These aged astrocytes show characteristics of the senescence-associate secretory phenotype (Salminen et al., 2011) and studies in AD animal models showed morphological changes in astrogial profiles (Olabarria et al., 2010; Yeh et al., 2011) . Surprisingly, we did not found differences in the morphological profiles of astrocytes in the subiculum (either when comparing AD to controls or when correlating with aging). These animal studies reported region-specific astroglial changes, with areas showing age-dependent hypertrophy, hypotrophy or unchanged astroglial profiles (Rodríguez et al., 2014). It is possible that in human aging and AD such variation exists. The meaning of the morphological changes in microglia and astrocytes, in aging and AD, is still to be understood. To best of our knowledge, this was the first studying addressing astrocyte morphological changes in human tissue. Further morphological studies, addressing other areas and their spatial relation to AD pathology (Aβ and tau), would be important to see if the findings of AD animal models replicate human pathophysiology. We should keep in mind that translation from animal models to human tissue analysis should be done carefully. Current animal models do recapitulate the full spectrum of the human disease, and key molecules in AD such as Aβ, tau, and ApoE are different between mice and humans in their sequences, pathogenicity or number of isoforms expressed (Onos et al., 2016; Sasaguri et al., 2017). Furthermore, there are noteworthy differences between mice and humans regarding microglial characteristics, in distribution, gene expression, and states of activation (Franco Bocanegra et al., 2017).

Cytokines are involved in nearly all aspects of neuroinflammation, including pro- and anti-inflammatory actions, bystander neuronal injury, chemoattraction, and response of

microglia to Aβ deposits. Microglia and astrocytes are the principal source of cytokines in AD (Heneka et al., 2015). Biochemical changes in the brain are often reflected in the CSF, because of the close contact of the CSF with the neuronal tissue (Blennow et al., 2010), and CSF analysis may provide insights into neurological disease pathways that may not be identifiable using blood or other biological fluids. It is comprehensible that due to growing evidence of neuroinflammation in AD pathogenesis, levels of cytokines and other inflammatory markers in fluids (CSF and peripheral blood) have been extensively investigated to uncover mechanisms of neuroinflammation in dementia or in the context of biomarker research (Brosseron et al., 2014) . Despite a huge amount of data obtained in different studies, results are conflicting and reveal significant heterogeneity in methodology (Brosseron et al., 2014; Lai et al., 2017; Saleem et al., 2015). In the last study of this Thesis, we sought to compare the profile of a large panel cytokines (pro- and anti-inflammatory) in the CSF of a group of AD patient´s according to their age and clinical characteristics. Similar to the first pathological study, we also wanted to know if neuroinflammatory changes in AD reflect common pathways associated with the process of neurodegeneration or differ from other disorders. For this question, a FTD group was included in the analysis. First, we analysed the clinical cohort by searching differences between the control group and the dementia group (AD and FTD cases). We found that the dementia group have significantly higher values of a broad number cytokines (IL-1ra, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-15, eotaxin, G-CSF, GM-CSF, MIP-1α, PDGF-BB, TNF-α). These data support the perception that the inflammatory process that accompanies neurodegeneration is a complex balance between different pro- and anti-inflammatory pathways. The simplistic view of inflammation in context of AD as having an exclusively detrimental or beneficial role is probably not correct in this complex and time dependent context. In fact, obtaining longitudinal data for inflammatory CSF markers (from prodromal to late stage AD) will be crucial to understand this dynamic. Subsequently, we searched for differences in AD and FTD, taking into account that the neuropathology and genetic basis of the two disorders are distinct. Nevertheless, we have to keep in mind that frontotemporal lobar degeneration is a heterogeneous disorder from the pathological point of view, with about half of the FTD cases showing TDP43 pathology and 40% tau pathology (Mann and Snowden, 2017; Taipa et al., 2012). We found that FTD cases had higher levels of

MCP-1 (CCL2), and AD cases higher IL-15 levels. Despite evidence of an up-regulation in AD brain tissue (Sokolova et al., 2009), the results regarding implication of MCP-1 in AD are contradictory, and there is no consensus about a positive or negative role of MCP-1 or its receptor on the pathophysiology of AD (Conductier et al., 2010; Sokolova et al., 2009). Furthermore, there are studies reporting elevated levels in CSF of AD and MCI patients (Brosseron et al., 2014; Galimberti et al., 2006) and others showed unchanged levels (Brosseron et al., 2014; Johansson et al., 2017; Kauwe et al., 2014). In contrast, there are limited studies in FTD patients, but the data available suggested upregulation in sporadic FTD (Galimberti et al., 2015). IL-15 is a proinflammatory cytokine and scarce information is available on the exact role of IL-15 in the neurological diseases, namely neurodegenerative dementia (Rentzos and Rombos, 2012). A small study found it increased in CSF from patients with AD and FTD (Rentzos et al., 2006), but more recently, increase IL-15 CSF levels were found only in GRN mutation carriers and not in sporadic FTD (Galimberti et al., 2015). Our findings suggest that these cytokines may have different roles in neurodegeneration in both conditions, at least in this stage of the disease. Again, longitudinal observations will be important in our understanding the roles in these disorders. Finally, we compared the AD group according to the age of onset (EOAD vs LOAD). Remarkably, we found that EOAD had lower values of IP-10 compared to LOAD cases. Additionally, the values of IP-10 and MIP-1β correlated positively with age in AD group. IP-10 has been shown to be upregulated in reactive astrocytes of AD brains and frequently associated to amyloid plaques (Xia et al., 2000). Interestingly, the same study showed that IP-10 is upregulated in a co-ordinated manner with another chemokine, MIP-1β. Thus, our results raise the possibility of an age effect in IP-10 dysregulation in AD pathogenesis. The possibility of an age effect on AD neuroinflammation process, due to either an aged proinflammatory status or dysfunctional senescent immune cells, must be taken into account when therapeutic approaches are designed or tested. Adding to the biological complexity of AD, we must be aware of the frequent co-occurrence of neurodegenerative pathologies in the brains of symptomatic and asymptomatic patients, particularly in older people (Rahimi and Kovacs, 2014).

In this study, we also found that cognitive status in AD correlated positively with a broad range of pro- and anti-inflammatory molecules. Furthermore, there was a negative correlation with disease progression in some of these cytokines. In vivo imaging studies using translocater protein-binding (considered a marker of microglial activation) showed that microglia activation was positively correlated with MMSE scores in AD patients, and that AD patients with slow decline had higher translocater protein-binding (considered a marker of microglial activation) (Hamelin et al., 2016). These findings are in agreement with our results, supporting the concept of a dynamic process that can have different consequences (protection vs injury) depending on the time and stage of disease.

In conclusion, the studies presented in this thesis call for a reappraisal of aging as modulator in sporadic AD associated inflammation. In LOAD, there are complex interactions between Aβ, degrading system dysfunction and inflammation. An age effect in the immune system can undoubtedly impact on degrading machinery and promote aberrant secondary inflammation (Zuroff et al., 2017). At the same time, *in vivo* imaging studies showed greater microglia activation (TSPO binding) (Kreisl et al., 2016) and widespread hypometabolism dysfunction (with the same degree of amyloid) in EOAD. In this Thesis we report differences in pathology, even at a late stage, and in the cytokines profile according to age of the subject. Given that immunotherapeutic approaches are currently the most advanced treatments for AD, our studies supports the idea that inflammation in the context of AD is not exclusively detrimental or beneficial, but has to be fine-tuned. The study of this delicate balance in the different ages will be important to understand treatment efficacy in clinical trials and eventually, not only direct treatment to early disease stages, but also the possibility of establishing different treatment approaches in light of the age of the patient.

5. Future perspectives

Although a major area of research in the last two decades, the precise biological changes that lead to AD are still poorly understood. Emerging evidence suggests that inflammation plays an important role in pathogenesis of the disease. Additionally, aging is associated to changes in the neuroinflammatory system that can lead to or modulate Alzheimer´s disease pathology. While the results presented and discussed in this thesis contribute to a better understanding of the subject, they also raise a number of questions that merit further investigation.

As discussed previously, longitudinal studies addressing neuroinflammation in AD will be of paramount importance to understand the dynamics of their contribution in the disease pathology (either protective or deleterious). Clarifying the temporal relationship between the multiple players of the inflammatory response in AD pathogenesis will be essential to identifying potential pathways for targeted anti-inflammatory treatments. The age of disease onset can unveil some differences in this longitudinal profile that must be taken into account when designing immunotherapeutic treatments.

From the pathological point of view, additional studies in cases with low Braak stages (with and without known cognitive decline) would be helpful in clarifying the temporal relationship of inflammatory pathology markers with AD pathology and age. In older age, co-occurrence of different neuropathologies should also be considered in the analysis. It will be important to understand if the morphological changes in microglia and astrocytes that have been elegantly shown in animal models of aging and AD, are also found in humans. This will allow in depth studies searching for the pathophysiological basis of such changes. We are currently doing further morphological studies in astrocytes in other areas in AD, but also in FTD cases. Furthermore, we still need more selective markers of the different microglia states and better ways to distinguishing activated microglia from infiltrating blood-borne macrophages. This will help to determine whether biological findings in the animal models are applicable to humans, and whether therapeutic approaches targeted to these models of disease predict treatment response in human diseases.

The alterations of cytokine levels reflect the disturbance of the immune system in AD, however, the evidence from body fluid is insufficient to decide whether these changes are initiating or a secondary event of the disease, and if they are protective or deleterious. As stated previously, longitudinal data will be crucial to understand the precise biological role of inflammation in disease pathogenesis. The current possibility of *in vivo* studies with imaging microglia markers together with AD pathology markers (Aβ and tau) will be very helpful. Nevertheless, continuous search for inflammatory players in disease pathogenesis and the study of their relation to clinical findings will increase knowledge and open areas for further research. The recent findings of a role in lipocalin in the inflammatory response in AD, led us to consider this marker and we are currently studying lipocalin levels in our clinical cohort (serum and CSF). Brain imaging analysis is also ongoing. This will help understanding at structural level the clinical correlations addressed in this thesis.

6. References

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7. Annexes

7.1. Does the Interplay Between Aging and Neuroinflammation Modulate Alzheimer's Disease Clinical Phenotypes? A Clinico-Pathological Perspective

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Review

Does the Interplay Between Aging and Neuroinflammation Modulate Alzheimer's Disease Clinical Phenotypes? A Clinico-Pathological Perspective

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Abstract.
Alzheimer's disease (AD) is a chronic neurodegenerative disorder and is the most common cause of dementia worldwide. Cumulative data suggests that neuroinflammation plays a prominent and early role in AD, and there is compelling data from different research groups of age-associated dysregulation of the neuroimmune system. From the clinical point of view, despite clinical resemblance and neuropathological findings, there are important differences between the group of patients with sporadic early-onset (<65 years old) and late-onset AD (>65 years old). Thus, it seems important to understand the age-dependent relationship between neuroinflammation and the underlying biology of AD in order to identify potential explanations for clinical heterogeneity, interpret biomarkers, and promote the best treatment to different clinical AD phenotypes. The study of the delicate balance between pro-inflammatory or anti-inflammatory sides of immune players in the different ages of onset of AD would be important to understand treatment efficacy in clinical trials and eventually, not only direct treatment to early disease stages, but also the possibility of establishing different treatment approaches depending on the age of the patient. In this review, we would like to summarize what is currently known about the interplay between "normal" age associated inflammatory changes and AD pathological mechanisms, and also the potential differences between early-onset and late-onset AD taking into account the age-related neuroimmune background at disease onset.

Keywords: Aging, Alzheimer's disease, inflammation, microglia, phenotype

INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disorder and is the most common cause of dementia worldwide. The two major neuropathological hallmarks of the disease are senile plaques, which are mainly composed of extracellular deposits of amyloid-β (Aβ) and neurofibrillary

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tangles, which consist of intracellular aggregates of aberrantly phosphorylated tau protein. This is accompanied by neuronal and synaptic loss, dendritic and axonal changes, and inflammatory reaction lesions [1, 2]. Cumulative data suggests that neuroinflammation plays a prominent and early role in AD [3–8]. Microglia cells are the predominant resident immune cells in the central nervous system (CNS) [9]. Recently, some studies highlighted the biological process of age-related changes associated with microglial cells [10–12] and suggest that microglial senescence can be directly associated to neurofibrillary degeneration [13]. From the clinical point of view, despite clinical resemblance and neuropathological findings, there are important differences between the group of patients with sporadic early-onset (<65 years old, EOAD) and late-onset AD (>65 years old, LOAD). Thus, it seems important to understand the age-dependent relationship between neuroinflammation and the underlying biology of AD in order to identify potential explanations for clinical heterogeneity, interpret biomarkers, and promote the best treatment to different clinical AD phenotypes.

In this article, we will discuss the current knowledge regarding the interplay between "normal" age associated inflammatory changes and AD pathological mechanisms. In addition, we will discuss the potential differences between EOAD and LOAD taking into account the age-related neuroimmune background at disease onset. We will give particular emphasis to microglia due to their predominant role in the immunological process within the CNS.

BRAIN IMMUNE SYSTEM

Microglia are the resident immune cells of the CNS and considered the tissue-resident macrophages. These cells were first described by Nissl in 1899, who distinguished microglia from other neural cells based on the shape and their nuclei [14]. Microglia cells arise from myeloid precursors and constitute an autonomous population distinct from the peripheral circulating mononuclear phagocytes [15]. These cells account for up to 16% of total cell CNS population and this is dependent on the brain region [9]. There is limited replication and turnover of microglia, suggesting that microglia are a very long-lived and stable cell population [9, 12]. Microglia can provide several macrophage-related activities that provide an innate immune response as the first and main form of active immune defense in the brain [9]. The term microglial activation encloses the process where microglia change shape, molecular signature, and cellular physiology in order to respond to injury or disease [16]. Resting microglia are characterized by a small cell body, highly ramified processes with weak expression of associated cell surface marker antigens [17]. In contrast, activated microglia display shortened and extensively branched processes and hypertrophy of cell body [18]. The definition of resting microglia does not mean a passive spectator in the healthy adult CNS. *In vivo* two-photon microscopy imaging studies showed that microglia survey the brain parenchyma by constantly extending and retracting their processes, and react rapidly to brain injury or insult, and are more properly termed "surveillant" [19–21]. The functions of microglia in the normal healthy brain beyond immune surveillance are unclear, but recently more sophisticated functions were described such as participating actively in the maintenance and plasticity of neuronal circuits and contributing to the protection and remodeling of synapses [22, 23].

Microglial activation states have been classically described as activated (M1) or alternatively activated (M2) [24]. The M1 phenotype is characterized by production of proinflammatory cytokines, such as IL-1β, tumor necrosis factor alpha (TNFα), and IFN-γ, whereas in the M2 phenotype microglia secrete anti-inflammatory cytokines, such as IL-4, IL-10, and transforming growth factor-β, which downregulate inflammation and promote tissue remodeling/repair and angiogenesis [25]. However, this categorizing system relays on peripheral macrophages studies, which do not recapitulate all microglial functions and is likely an oversimplification [21].

The second type of neuroimmune cells is the perivascular macrophages [26]. They seem to be derived from circulating macrophages, and are able to perform all the known functions of peripheral macrophages; they undergo complete turnover approximately every 3 months [27, 28]. Finally, the circulating blood monocyte can enter the CNS, but it is not clear how often it happens under non-inflammatory conditions. In conditions of disrupted blood-brain barrier, and when properly stimulated, they can differentiate into microglia-like cells or perivascular macrophages morphologically and phenotypically [26].

Astrocytes are the most abundant glial cells in the CNS and their function is critical for the support of neuronal homeostasis. The term astrogliosis describes a wide range of both molecular and func-

tional changes in astrocytes aimed to neuroprotection and repair of injured neural tissue [29, 30]. Recently it has been shown that that reactive astrogliosis and glial scar formation play essential roles in regulating CNS inflammation [29]. Reactive astrocytes in response to different kinds of insult can produce molecules with either pro- or anti-inflammatory potential. Additionally, reactive astrocytes can exert both pro- and anti-inflammatory effects on microglia [31, 32].

NEUROINFLAMMATION IN BRAIN AGING

There is clinical and experimental evidence that neuroinflammation in the aged brain is characterized by a shift toward a pro-inflammatory state [9, 33]. *In vivo* imaging studies using ^{11}-C-*R*-PK1195 PET ligand, which is upregulated in activated microglia cells, showed an increase in the specific binding with age in several cortical and subcortical structures, indicating that activated microglia gradually appear in the aging human brain [34]. In parallel, age senescent alterations can contribute to a dysfunctional microglia [12, 35, 36]. In the next paragraphs, we will address these apparent competitive perspectives of age-related neuroinflammation.

Inflammation in the brain is defined by upregulated astrocyte and microglial cell reactivity in association with increased levels of circulating cytokines such as TNFα, IL-1β, and IFN-γ [37–39]. With aging, microglia phenotype shifts progressively toward the activated form, together with enhanced sensitivity to inflammatory stimuli (priming phenomena) [9, 40]. In normal human brain aging, microglia is characterized by upregulation of glial activation markers such as IL-1α [41] and major histocompatibility complex II (MHC II) [42]. MHC II is important because it is conserved across species and is interpreted to indicate microglial priming [9]. There is compelling evidence from different research groups and aging models, that following different types of challenge (bacteria, virus, stress, surgical intervention), aged animals exhibited a clear and exaggerated neuroinflammatory response, when compared to young adult animals [33, 43–46]. These studies provided evidence that during lifespan, episodes of systemic inflammation and cytokine stimulation can "instruct" microglia and increase their reactivity [23, 33]. Interestingly, some of these sensitized neuroinflammatory responses are specific to the hippocampal formation, which is important for memory function [33]. Microglia from the aged CNS could be described as hyper-vigilant to disturbances in central homeostasis with less capability of shifting among functional states.

Proteins expressed in CNS microenvironment, which are known to inhibit microglia activation or pro-inflammatory immune responses, were implicated in the mechanism how microglia becomes chronically sensitized during normal aging [47]. In fact, some lines of research describe various proteins that activate anti-inflammatory signals following ligand receptor interactions [48], particularly CD200 [49–51] and fractalkine (CX3CL1) [51–53]; interestingly, both are preferentially expressed in neurons. These proteins inhibit microglia through their cognate receptor, which is expressed predominantly in myelomonocytic cell types. During aging, the expression of levels of these ligands decreases concurrently with increases in microglial activation status. More recently, another line of research suggests that significant and prolonged elevation in hippocampal corticosterone (the endogenous glucocorticoid in rodents) leads to microglial priming [51]. However, the simplistic view that aging CNS shifts microglial polarization from alternative M2 state to the classical, proinflammatory state, should be interpreted cautiously because many studies found that both M1 markers and M2 markers are increased in aged mice [12]. For example, active microglia from aged mice actually had higher levels of IL-10 production (an anti-inflammatory cytokine) than those of adult mice and lower expression of TGFβ (an inflammatory cytokine) [54]. In this case, the maintenance of inflammatory response could be attributed to an impaired response to IL-10 in the aged brain [9]. Furthermore, primed microglia phenomena have been described mainly in mouse models [9, 55], and less in human brain research [56]. More recently, research studies showed that the cerebrospinal fluid (CSF) levels of YKL-40 (a microglial marker) increase in normal aging [57–59].

Together with this perspective that microglia becomes primed and more reactive with age, others showed that microglia becomes senescent and less reactive with age [10, 11, 13]. In the healthy young CNS, microglia have a typical ramified morphology and are distributed throughout the neural parenchyma in a "space-filling" manner [60]. Due to the prolonged lifespan of CNS microglia, they are more susceptible to accumulate aging-related changes [61], such as in the distribution, morphology, and behavior [12, 60] (Table 1). Many microglial cells in the aged brain show dystrophic features indicative of age-

Table 1
Summary of principal changes associated to microglial aging (adapted from Wong [60] and Wyss-Coray [6])

Changes in microglial distribution
Replicative senescence (reduced mitotic activity in response to CNS injuries)
Decreases in regularity in distribution
Changes in morphology
Decrease in individual microglial ramification (dendritic arbor area, branching, and total process length)
Appearance of morphological changes suggestive of increase activation state (shortened and extensively branched processes and hypertrophy of cell body)
Appearance of dystrophic microglia (deramified, fragmented, or tortuous processes, cytoplasmic beading/spheroid formation)
Changes in microglial dynamic behavior and function
Decrease in the motility and migration process
Changes in intercellular signaling and marker expression (MHC II, CD11b)
Impaired phagocytosis
Impaired proteostasis

related alterations. This dystrophic microglia have de-ramification or decrease arborization of their processes, loss of finely branched cytoplasmic process, cytoplasmic beading/spheroid formation, and shortened and twisted cytoplasmic processes, and in some instances there is partial or complete cytoplasmic fragmentation [38]. The meaning of these morphological changes or why they happen is still to be understood.

Age-related changes were also described in astrocytes, particularly emphasizing that aged astrocytes show characteristics of the senescence-associated secretory phenotype, which involves increased secretion of inflammatory components [62].

In summary, aged microglia are primed with exaggerated and prolonged responses to inflammatory stimuli and also display dysfunctional dystrophic age associated features. Yet, it is still to be determined if microglia activation is the cause of neurodegeneration or a secondary reactive (beneficial) process; or if the neurodegeneration is actually secondary to microglia senescence and associated loss of microglial protection.

NEUROINFLAMMATION IN ALZHEIMER'S DISEASE

After two decades of the amyloid cascade hypotheses proposed by Hardy and Higgins [63], multiple lines of research still support the Aβ aggregation as the critical step that initiates AD pathology. However, despite required, it seems that Aβ aggregation is not sufficient for the development of the neuropathological and clinical syndrome of AD [64]. Several research studies report links between AD and genes regulating immunity as well as the expression of immune factors in blood, CSF, and brain tissue [8, 65–68]. There is compelling data that neuroinflammation in AD is not a passive mechanism activated by senile plaques and neurofibrillary tangles, but instead contributes, as much or even more, to pathogenesis as do plaques and tangles [65, 68, 69]. Epidemiological studies indicate that systemic markers of the innate immunity are risk factors of LOAD [70–73] and more recently, inflammation in AD gained strong support from genome-wide association studies that identified genes involved in inflammation that are associated with increased risk of developing AD [74], including TREM2 [75, 76] and CD33 [77, 78]. Prospective cohorts' studies suggested that elevations in inflammatory mediators may be present years before clinical disease onset [70, 79, 80]. However, other longitudinal studies did not report associations between inflammation and AD risk [81, 82]. Furthermore, non-steroidal anti-inflammatory drug (NSAID) epidemiology and clinical trials showed mostly negative results, playing against the importance of inflammation in AD pathogenesis. However, these disappointing results are no surprising taking into account that normal physiological cytokine regulation of glia activation and microglial phenotypes are highly dependent of the context and the disease stage [65]. More recently, studies have consistently found an increase in CSF YKL-40 levels in AD. They also found a correlation between CSF YKL-40 levels with markers of neurodegeneration, such as tau, and with at-risk $\varepsilon 4$ carriers during mid middle age [57–59].

Neuropathological studies have shown the presence of a broad variety of inflammation-related proteins (complement factors, acute-phase proteins, proinflammatory cytokines) and clusters of activated microglia around amyloid plaques (Fig. 1) in AD subjects and also AD mice models [8], and these findings have been implicated in the neurodegeneration pro-

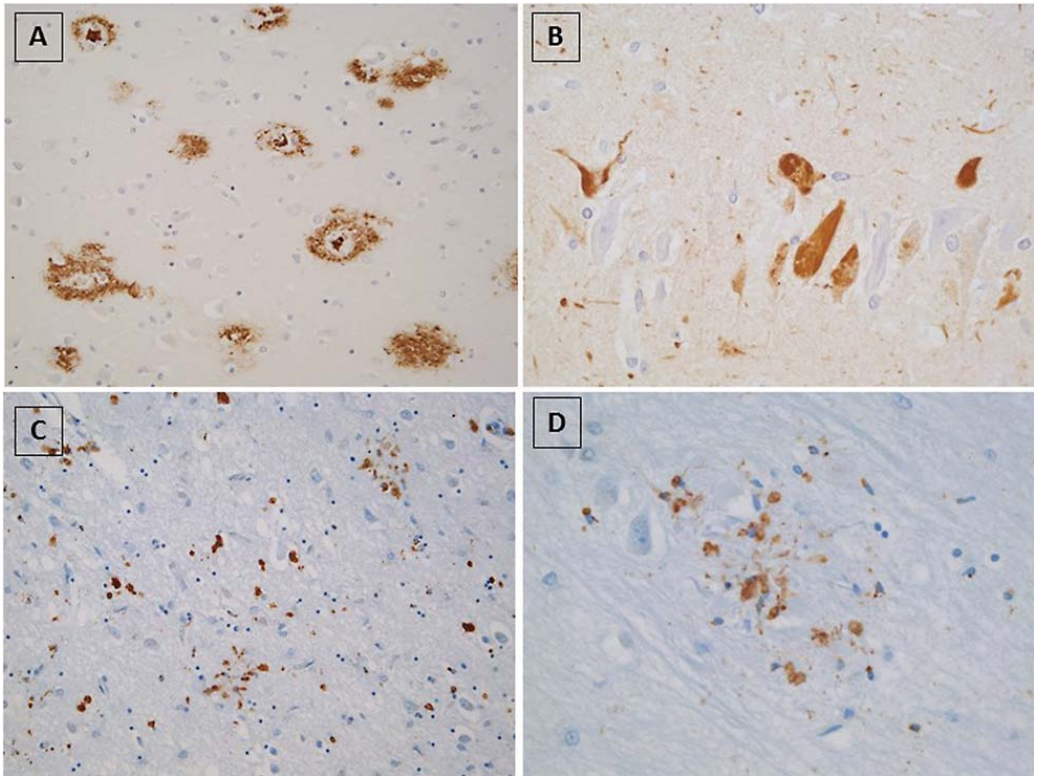

Fig. 1. Alzheimer's disease neuropathology. A) Senile plaques and globose diffuse deposits demonstrated with anti-Aβ antibody (M 0804, Dako). B) Neurofibrillary tangles demonstrated by phosphorylated tau protein immunohistochemistry (PHF-Tau; AT8, Thermo Scientific). C) Diffuse distribution of activated microglia in the cortex with clustering within and around amyloid plaques. D) Higher magnification of amyloid plaque with activated microglial in the CA4 region of the hippocampus (C and D: CD68 immunohistochemistry; PGM1 clone, Dako).

cess [4, 83]. Neuropathological studies also showed that the neuroinflammatory response in the neocortex is present in the early stages of AD pathology and precedes the late stage, tau-related pathology [84]. Furthermore, microglial activation has been shown to progress with the clinical stage of dementia, with neuropathological stage of disease severity, and with stage of progression of Aβ plaques [67, 85, 86]. *In vivo* imaging studies, using ^{11}-C-*R*-PK1195 PET ligand, showed that activated microglia accumulate near the amyloid plaque pathology, and that activated microglia burden correlates with cognitive decline [87].

The pathological accumulation of Aβ is considered the key factor that drives neuroinflammation responses in AD [65]. The chronic deposition of Aβ stimulates the persistent activation of microglial cells in AD [88]. Microglia undergoes a progressive switch from a neuroprotective M2 status to a classically activated phenotype M1, characterized by production of proinflammatory cytokines [89]. The persistent microglia activation and consequently microglia-derived cytokine overexpression, caused by continuous formation of Aβ and positive feedback loops between inflammation and amyloid-β protein precursor processing, can increase Aβ production and decrease Aβ clearance, ultimately causing neuronal damage [65, 86, 89]. In addition, ongoing exposure to Aβ, chemokines, cytokines, and other inflammatory mediators can be responsible for the functional impairment of microglial cells seen at plaque sites [11, 90] and thus impede the protective role of microglia in Aβ clearance [91]. Recently, Kim et al. [92] showed that soluble Aβ oligomers impair synaptic plasticity and cause synaptic loss in mouse AD models and brains of AD patients binding to the murine PirB (paired immunoglobulin-like receptor B) and its human ortholog LilrB2 (leucocyte immunoglobulin-like receptor B2) receptors, respectively. The PirB receptor was first described exclusively in the immune system but is now know to be expressed by neurons.

Microglia can have different roles and effects depending on the particular disease stage and which brain region is affected in each model [65]. AD mouse models studies showed that in younger ages, together with the appearance of the first Aβ plaques, the microglia is activated toward the alternative state and at older ages, together with the increased accumulation of extracellular oligomeric Aβ, there

is a widespread microglial activation toward the classic phenotype [93]. Recently, Sudduth et al. described that in the early-stage AD brains there is an apparent polarization toward either M1 or M2 brain inflammatory states [94]. The M2 polarized group had great number of neuritic plaques, eventually reflecting disease progression. The heterogeneity found in the early stage AD can influence the response to therapeutic agents that act on immune system and inflammation [94]. The neuropathological study of AD patients that had undergone active Aβ vaccination as part of the AN1792 trial showed significantly reduced levels of Aβ and reduction of aggregated tau in neural processes (not in neurofibrillary tangles), and, although there was no difference on total microglial load, there were reduced levels of a range of activated microglial species when compared to patients who died from AD without treatment [95, 96]. These findings suggest that downregulation of microglial activation through Aβ immunotherapy possibly reduces the inflammatory component of the neurodegeneration of AD [95]. However, a different line of research supports that aging-related microglial degeneration and loss of microglial neuroprotection rather than microglial activation contributes to the onset of sporadic AD [11]. A role for peripheral-derived macrophage cells in AD pathophysiology have recently come under attention [97]. There is extensive evidence that blood-derived monocytes can phagocytose Aβ [98] and that these cells can be recruited to the AD brain, albeit in low numbers [99].

Reactive astrocytes tend to accumulate around fibrillar amyloid plaques [100]. Similar to microglia, astrocytes release cytokines and other potentially cytotoxic molecules after exposure to Aβ thus aggravating the neuroinflammatory response [65]. Glial cell activation can be an early event in AD process, even preceding Aβ deposition. Recently, Rodriguez-Vieitez and colleagues [101], using a PET tracer for astrocytes (^{11}C-deuterium-L-deprenyl), showed prominent initially high and then declining astrocytosis in autosomal dominant AD carriers, contrasting with the increasing Aβ plaque load during disease progression. This study provided *in vivo* evidence that astrocyte activation is a very early feature of, at least familial, AD pathology [101]. Other lines of research have linked senescent astrocytes to the increase risk of sporadic AD [102].

In summary, the role of microglia remains controversial in AD pathogenesis and the question of whether activated microglia aids in promoting clearance of toxic Aβ species or if their proinflammatory profile exacerbates pathology is currently a topic of debate [103]. Although there is broad evidence of a large immune response component in AD, the issue of which activation phenotype affects the onset or progression of the disease and, consequently, which should be the therapeutic target remains to be determined [104]. Furthermore, the questions regarding the role of excessive astrogliosis or astrocyte senescent loss of function in AD pathogenesis remains to be solved [100].

EARLY AND LATE-ONSET ALZHEIMER'S DISEASE

Regardless of the clinical resemblance and neuropathological findings, important differences between EOAD and LOAD patients have been reported. The separation of EOAD from LOAD at 65 years old is a conventional cutoff point indicative of a sociological partition in terms of employment and retirement, but there is no specific biological significance to use this specific age, and there is a range of disease features that do not respect this arbitrary division [105, 106]. However, this arbitrary cutoff point has been used widely by different research groups and allowed the uniform study of AD patients with different ages of onset.

Clinical presentation

Whether age of onset defines the clinical presentation of AD has been a matter of debate for decades and reports on this issue are often contradictory. Nonetheless, some differences have been consistently recognized. Earlier onset is associated with a worse prognosis and a faster progression. Younger-onset patients have comparatively worst outcomes in the Mini-Mental State Examination at baseline, show a steeper cognitive and functional decline, and seem to have higher mortality risks when compared to older-onset patients [107–109]. In addition, different patterns of cognitive deficits are apparent; non-amnestic presentations are more often found in early-onset disease, described in 33–64% of EOAD compared to 6–12.5% of LOAD patients [105, 110].

Earlier neuropsychological studies have shown that younger patients have more language disability when compared to older-onset patients [111–113]. The risk of having language difficulties detected by caregivers has also been shown to nearly duplicate for each 10-year decrease in AD patients' age [114]. Other groups have recognized a greater impairment in

measures of attention, praxis, and visuo-contruction tasks in EOAD [115–117]. On the other hand, LOAD patients seem to consistently have preferential memory involvement [118–120]. To explore the relation between this clinical duality and pathologic features, Murray et al. [121] divided a cohort of AD patients into "hippocampal sparing", "limbic predominant", and "typical AD" according to neurofibrillary pathology distribution. They have shown that a younger age of onset (mean 63 years) was associated with greater neurofibrillary tangle burden in cortical association areas and that older age (mean 76 years) was more often associated with limbic predominant pathology. The hippocampal sparing group had greater prevalence of atypical presentations and a faster cognitive decline, similar to what has been described in EOAD.

Seizures and extrapyramidal features seem to be more frequent in EOAD [111, 122]. There are contradictory reports in other symptoms in both groups. For example, there are reports of higher anxiety levels in EOAD [123], while others have shown greater neuropsychiatric and behavioral symptoms in LOAD, including anxiety, depression, agitation, hallucinations, and delusions [124, 125].

Limited research has been reported into sex differences in brain aging, particularly neuroinflammation process. However, gender effect is an interesting issue due to the differences of the neuroendocrine milieu and its possible relation to inflammation cascades (particularly steroid-related pathways). The dynamic change in hormonal status in women during the menopause transition may promote a dysregulation of cellular processes involved in hypothalamic-pituitary-adrenal axis and thus have potential implications on stress-mediated neurotoxicity [126]. It is also important to recognize the importance of immunological differences in males and females within the CNS at different development time points and their possible relevance for the susceptibility in the development of neurological conditions later in life [127]. A recent work in mice by Mangold and colleagues showed a greater induction of MHC class I components and receptors with aging with this finding being greater in females than in males [128]. However, despite the prevalence of AD being greater in women, the prevailing view has been that this difference is due to the fact that women live longer than men on average, and older age is the greatest risk factor for AD. Many studies of incidence of AD have found no significant difference between men and women in the proportion who develop AD at any given age [129].

Biomarkers

Magnetic resonance imaging (MRI) studies show that younger-onset patients have greater generalized neocortical atrophy than LOAD subjects when compared to healthy controls [118, 130]. This is in accordance with glucose metabolism studies, which demonstrate a premature decline in glucose metabolism and a more severe and widespread hypometabolism in EOAD [131]. Regarding regional differences, older patients tend to have a more circumscribed involvement, with preferential reduction in the hippocampus and related structures, including the amygdala [132] and retrosplenial and temporoparietal junction volumes [130], while younger patients tend to have a greater temporoparietal and parietooccipital grey matter atrophy [115, 120]. White matter atrophy mimics this pattern [133]. Moreover, both perfusion and glucose metabolism studies have shown a predilection for temporo-parietal-occipital association areas in EOAD versus medial temporal cortex susceptibility in LOAD [119, 134]. Interestingly, another study has shown no significant difference in total or regional amyloid burden, measured by Pittsburg compound-B PET, despite showing decreased glucose metabolism in bilateral temporoparietal and occipital cortex in EOAD. This finding suggests that both early Aβ and increased susceptibility to pathology in younger onset patients might be responsible for cortical dysfunction in EOAD [135]. The greater involvement of hippocampal-related structures in LOAD is also apparent in functional connectivity studies that have shown that older patients have decreased activation of the anteromedial temporal network, correlating with poorer performance in memory tasks; EOAD was associated with less activation of the dorsolateral prefrontal network, manifested by worse performance on executive function tasks [118].

CSF pathophysiological markers for AD include decrease levels of $A\beta_{1–42}$ and increase levels of total tau and hyperphosphorylated tau. The use of these biomarkers combined is associated with significant sensitivity and specificity in the diagnosis of AD [136]. There is some evidence that EOAD patients have a greater reduction of $A\beta_{1\text{-}42}$ (and corresponding greater elevation of tau) than LOAD patients when compared to young and old controls, respectively, although no differences emerge in the direct comparison between EOAD and LOAD [137]. Others have reported lower levels of $A\beta_{1\text{-}42}$ in EOAD [138] or no differences [120, 139]. A study comparing CSF biomarkers along different EOAD subtypes,

including amnestic, logopenic progressive aphasia and posterior cortical atrophy found no differences in the Aβ levels, but showed that posterior cortical atrophy had lower levels of total tau and phosphorylated tau [140].

Genetics

Amyloid precursor protein, presenilin 1, and presenilin 2 mutations can cause autosomal dominant AD, and although they may be present in up 71% of familial cases, they account for only about 1–5% of all AD patients. These patients typically have an early or very early-onset disease (<45 years) [136, 141, 142]. A well-recognized genetic risk factor for AD is the APOE ε4 allele. It is usually associated with greater hippocampal atrophy and a poorer performance in memory based tasks [121, 142] and it decreases the age of onset by up to 2.45 years for each copy of the allele [142, 143]. Conversely, non-APOE ε4 patients tend to have greater structural and clinical involvement of non-hippocampal, neocortical areas [121]. ApoE ε4 allele carriers among AD patients are most frequently found in the 60–69-year-old range [144], therefore including both older EOAD patients and younger LOAD patients. The ApoE ε2 allele is less frequently found in AD patients than in normal controls and there seems to be no difference in its prevalence between EOAD and LOAD [144]. Genome wide association studies have identified several other risk genes for LOAD. The association between nine of them (PICALM, CLU, CR1, BIN1, CD2AP, EPHA1, MS4A4A, CD33, and ABCA7) has been shown to account for 1.1% of age of onset variation, versus 3.9% of variation provided by ApoE. The most significant association was found for the CR1, BIN1, and PICALM genes [143]. Another candidate gene that may have an impact on age of onset is DCHS2, a gene expressed in the cerebral cortex [145]. Yet, and surprisingly, these genetic variants do not seem to bring significant value for the distinction between EOAD and LOAD, as they simply seem to anticipate pathology.

INTERPLAY BETWEEN BRAIN AGING, NEUROINFLAMMATION, AND AD PHENOTYPES

AD prevalence is strongly associated with increasing age and aging changes in microglia have been hypothesized to play a prominent role in disease pathogenesis [60]. Recently, the consistent pattern of increases in YKL-40 level with aging supports the concept that neuroinflammation is a process that occurs normally with aging [57–59]. The additional finding of a stronger association with at-risk ε4 carriers during mid middle age suggests that this age-related process may be further exacerbated in the presence of insults including amyloid deposition and neuronal injury [59]. There are important clinical differences between sporadic EOAD and LOAD. Taking into account the data regarding the importance of neuroinflammation in the pathogenesis of AD, particularly the role of microglia, and the differences of the neuroimmunological milieu of the aged brain, it is conceivable that the neuroinflammation associated to the AD can, at least in the beginning, differ between these two groups and contribute for the clinical differences. Not many studies have addressed this issue.

Hoozemans et al. [146] compared the presence of microglia and astrocytes, in clinically and pathologically confirmed AD and non-demented control cases in relation to age. In their study they suggested that the association between neuroinflammation and AD is much stronger in relatively young patients as compared to the older patients (age at death <80 versus >80 years old). Microglial activation increases with the neuropathological stage and disease severity [67, 85]. A key issue would be to know if inflammation differs between these two groups (EOAD versus LOAD) at different pathological and clinically AD stages.

Another remarkable finding is that, in contrast to AD, activated microglia is not found in the similar-appearing Aβ diffuse deposits of the brains of neurologically normal elderly individuals [147]. One of the possibilities is that for those unusual elderly individuals with only diffuse Aβ deposits there is an inherent difference in the responsiveness of microglia [86]. Interestingly, plaque-associated microglia were not seen in diffuse plaque-only young Down's syndrome brain [148]. This subgroup of cases was from very young patients (between 12 and 29 years old), possible supporting the notion that Aβ inflammatory response can also differ in the very young. More recently, a study showed that in Down's syndrome patients with AD pathology (>40 years old), there is a distinct neuroinflammatory phenotype compared to sporadic AD due to microglia bias toward an M2b phenotype [149]. Clinicopathological studies from brain donation programs showed that the presence of moderate and severe AD type pathology changes is more associated to dementia in younger old persons

than in older old persons [150]. These findings suggest that additional factors are involved in the clinical expression of dementia in the oldest old, such as variable tolerance to neuropathological lesions [150]. We speculate that different neuroinflammation apparatus in this age can partial explain this discrepancy.

The study of inflammatory cytokines in CSF as biomarkers of AD has shown very different and contradictory results between different research groups [89]. The analysis of different neuroinflammation-related proteins in the blood, including several interleukines (IL-1α, Il-1β, IL-6, IL-10), α2-macroglobulin, brain-derived neurotrophic factor (BDNF), complement factor H, and heat shock protein 90 (Hsp90) has not shown significant differences between EOAD and LOAD, but studies are scarce and with small samples [151, 152]. TNFα levels have been shown to be both higher and lower in EOAD [152, 153].

Some of the risk loci in modifying age of disease onset identified in genome wide association studies have recognized roles in the immune system, including phagocytosis and immune cell trafficking [154]. Both CLU and CR1 encode for proteins that regulate complement activation; EPHA1, mostly expressed in leukocytes, is involved in T cell regulation; ABCA7 is highly expressed in the hippocampal neurons and in microglia and is involved in Aβ processing; and CD33, overexpressed in AD patient's microglia, encodes for an endocytic receptor that takes part in cell-cell interactions and in immune cell regulation [154, 155]. TREM2, another loci associated to increase risk for AD identified, is involved in immune response [75]. There are studies that found a significantly earlier symptom of onset in patients with TREM2 variants [156], but others found only an association to shortened disease duration and not to age of onset [76]. Aβ cerebral amyloid angiopathy (CAA) and particularly Aβ related angiitis (ABRA), is other AD related clinical feature that bridges AD, inflammation and age. CAA describes a group of biochemically and genetically diverse disorders, which have in common the deposition of amyloid in media and adventitia of cortical and leptomeningeal vessels [157]. Sporadic CAA and AD have overlapping biology with shared risk factors [158]. Aβ vascular deposition affects about 30% of the otherwise normal elderly and over 90% of those with AD, in whom CAA tends also to be more severe [157, 159]. ABRA is characterized by a vasculitic transmural, often granulomatous, inflammatory infiltrates affecting leptomeningeal and cortical vessels

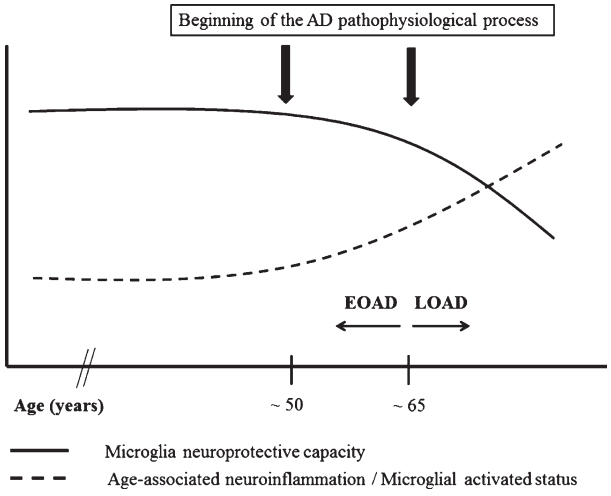

Fig. 2. Diagram illustrating age associated microglia dynamics and temporal Alzheimer's disease onset. Arrows exemplify two time points for the beginning of AD biomarkers [Aβ accumulation (CSF/PET), sequentially followed by tau-mediated neuronal injury (CSF)] at the preclinical stage.

that have abundant Aβ deposition within the vessel walls [159, 160]. The recent finding of autoantibodies against $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ forms of amyloid in the CSF of two patients with ABRA and inflammation associated to CAA [161, 162], together with the description of meningoencephalitis caused by active or passive immunotherapeutic approaches to reduce Aβ burden in AD [163], suggests that an immune response directed against Aβ may represent a common disease mechanism shared by ABRA and in complications of therapy for AD [160]. The mean age of presentation of ABRA is lower than that of sporadic non-inflammatory Aβ-related CAA (66 versus 76 years, respectively) [159, 160]. These findings support a role for the interactions between age, and inflammation in AD related pathophysiology and clinical expression.

In summary, the pathophysiological mechanisms underlying the clinical differences between EOAD and LOAD are still not well known, but the differences of neuroinflammation characteristics with aging can help to partially explain it (Fig. 2).

CONCLUSION

Understanding both sides of microglial and astrocytosis inflammation process at functional and molecular level will be necessary for the development of treatment strategies for AD and aging [12].

Additionally, the study of this delicate balance in the different ages of onset of AD would be important

to understand treatment efficacy in clinical trials and eventually, not only direct treatment to early disease stages, but also the possibility of establishing different treatment approaches in light of the age of the patient. The boost on AD diagnostic biomarkers will increase diagnostic certainty in life for the diagnosis of dementia with AD pathology. This refinement will allow the increased recognition of the more often atypical clinical presentations in EOAD and thus increase the knowledge (epidemiology, clinical progression, biomarkers studies, neuroinflammation associated process, etc.) for a possible better understanding of this complex disorder.

DISCLOSURE STATEMENT

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