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

Authors and Affiliations
Ricardo Taipa¹,²,³, Vitor Ferreira²,³, Paulo Brochado¹, Andrew Robinson⁴, Inês Reis⁵, Fernanda Marques²,³, David M Mann⁴, Manuel Melo Pires¹, Nuno Sousa²,³
¹ - Neuropathology Unit, Department of Neurosciences, Centro Hospitalar do Porto, Portugal
² - Life and Health Sciences Research Institute, University of Minho, Portugal
³ - ICVS/3B’s Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal.
⁴ - Division of Neuroscience and Experimental Psychology, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Salford Royal Hospital Foundation NHS Trust, Salford, UK

Corresponding author:
Ricardo Taipa, M.D.
Neuropathology Unit, Department of Neurosciences
Centro Hospitalar do Porto – Hospital Santo António
Largo Prof. Abel Salazar, 4099-001
Porto, Portugal
Email: rtaipa.neuropat@chporto.min-saude.pt; Phone: +351 222077500 (Ext 1620)

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Short running title: Microglia activation and astrogliosis in young and late onset Alzheimer’s disease.

Abstract
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 GFAP) 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.

List of abbreviations: AD – Alzheimer’s disease; Aβ – amyloid β; DG – dentate gyrus; EOAD – early onset AD; GFAP – glial fibrillary acidic protein; LOAD – late onset AD; NFT – neurofibrillary tangles.
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 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 of 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 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 GFAP was performed using the Ventana OptiView DAB IHC detection kit and the Ventana BenchMark Ultra processor (Ventana, Tucson, AZ). Sections of temporal (including hippocampus and parahippocampal region), frontal, parietal and occipital cortices were cut at 6μm 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 minutes at 36°C and GFAP (Z 0334, 1:2500, Dako) for 12 minutes at 36°C. Subsequently, the slides were incubated with OptiView HQ linker for 8 minutes, and then with OptiView universal HRP multimer (Ventana) for a further 8 minutes at 36°C. Tissue sections were incubated with OptiView universal DAB chromogen (Ventana) for 8 minutes 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) using a series of random temporal cortical sections across the groups (sixteen cases).

Microscopic semi-quantitative analysis

Microglia

Both CD68 and Iba1 antibodies mark microglial cells, both in resting and activated states (Figure 1A and 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 x20 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 (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 to 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 (5 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 40x oil-immersion objective. The subiculum and entorhinal cortex were selected and inside it, two smaller areas were designated for cell counting. Square probes (50x50 μm) 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, Vermont, 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 100x 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). A p-value of less than 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 microglia scores or astrogliosis in each group.

Results

Four groups were established (Table 1) based on confirmation from pathology (AD versus 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 =

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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 versus 7.6 years, respectively). The groups were not homogeneous regarding to gender with over representation of males in EOAD group. (See supplementary table 1 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 1C). Similarly to other descriptions, activated microglial cells were also 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 (supplementary figure 1). 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 $\tau$ coefficient; supplementary figure 2).

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 1D and 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.
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 ($r=0.650$, $p<0.01$) (supplementary figure 3). 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 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 4A). In the subiculum, only the EOAD had higher microglial scores than their aged matched control group (CONTy) ($p = 0.001$) (Figure 4B). Post-hoc testing in the hippocampal white matter did not show differences between any of the groups (Figure 4C). 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 4D). 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 4E).

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 4F). 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 CONTy, $p = 0.045$) (Figure 4G). Post-hoc testing did not show differences between any of the groups in the occipital cortex (Figure 4H).

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 4I). 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 5A). 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 5B). In CA4 post-hoc testing did not show differences between any of the groups (Figure 5C). 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 5D and 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 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 5F – 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 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 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.

The present 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 early onset and late onset AD. 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 the present study was to assess the degree of neuroinflammation according to age in AD, and not an association with AD pathological severity.

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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 versus 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. 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 [42], several studies indicate that astrocyte-mediated inflammatory process also contribute to neurodegeneration in AD [43]. Enhanced expression of GFAP and astrocyte hypertrophy have been identified in post-mortem tissue from AD cases [27,44,45]. 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 [46,47]. In old rodents, an increase, a decrease and no change in the number of GFAP positive astroglial cells have been reported [48]. 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 maybe 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) [49]. 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 [50]. In transgenic AD animals studies, astrocyte atrophy in both hippocampus (CA1 and dentate gyrus) [51] and entorhinal cortex was reported [52]. While astroglial atrophy appears as a generalized process, the astrocytes that surround plaques were hypertrophic with increased surface and volume of GFAP-immunostained profiles [51]. 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 [53] 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 aging 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 [54,55]. It is also possible that, similar as reported in animal models [50], there are region-specific astroglial changes in human aging and AD. Additionally, the analysis of astrocyte morphology in relation to proximity of AD pathology (Aβ and tau) could also be informative [51]. 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. 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 [56] and potential stronger inflammatory dysregulation in woman [57], it would be important to explore gender differences in AD associated inflammation and aging.

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 aging, 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 [58].

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Authors roles
Ricardo Taipa provided study design, did all the microscopical assessments, data analysis and wrote the paper.

Vitor Ferreira did the morphological astrocytes reconstructions.

Paulo Brochado did all the immunohistochemistry studies.

Andrew Robinson prepared sections for staining and immunohistochemistry.

Inês Reis prepared sections for staining and immunohistochemistry.

Fernanda Marques helped with discussions and paper writing.

David M. Mann helped with discussions and paper writing.

Manuel Melo Pires helped with discussions and paper writing.

Nuno Sousa provided supervision of the study design, helped with discussions and paper writing.

The authors do not report any conflicts of interest.
Table 1. Clinical and pathological status, demographic information and disease duration.

(NA, not applicable)

<table>
<thead>
<tr>
<th></th>
<th>Male : Female</th>
<th>Mean age at death (SD)</th>
<th>Interval of age at death</th>
<th>Mean age of disease onset (SD)</th>
<th>Mean disease duration (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOAD n = 19</td>
<td>17M : 2F</td>
<td>66.74 (± 6.62)</td>
<td>45 - 73</td>
<td>56.72 (± 6.11)</td>
<td>9.78 (± 2.46)</td>
</tr>
<tr>
<td>LOAD n = 11</td>
<td>4M : 7F</td>
<td>82.91 (± 5.13)</td>
<td>76 - 89</td>
<td>76.0 (± 4.59)</td>
<td>7.60 (± 1.26)</td>
</tr>
<tr>
<td>Controls Young n = 15</td>
<td>7M : 8F</td>
<td>59.47 (± 8.73)</td>
<td>38 - 71</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Controls Old n = 12</td>
<td>6M : 6F</td>
<td>83.75 (± 6.81)</td>
<td>75 - 95</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Legend to figures

Figure 1.

Ramified (a) and activated (b) microglia cells, as seen in CD68 immunostaining in temporal cortex (a – CONTy case; b – EOAD case). Clustering of activated microglial cells within an amyloid plaque (c, EOAD case). Astrocytes immunoreactive for 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 and d), 100 µm (b and e), 1mm (f).

a – c: CD68 immunohistochemistry (PGM1 clone, Dako); d – f: GFAP immunohistochemistry (Z 0334, Dako).
Figure 2.

Representative examples of the microglial and astrocytosis assessment according to the scale description. Scale bars: 100 µm (CD68) and 200 µm (GFAP).

CD68 (PGM1 clone, Dako) and GFAP immunohistochemistry (Z 0334, Dako).

Figure 3.

Representative examples of CD68 and 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).

Figure 4. Comparison of microglia scores between groups in different regions (bar graphs) and correlation between microglia severity score in occipital cortex and age at death (bottom right figure; $r = 0.322; p < 0.05$).

Bar graphs: *p<0.05 and **p<0.01, Kruskal-Wallis. Y axes represent semi-quantitative scale mean and bars represent ± SE.

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

Figure 6. Bar graphs representing GFAP surface, volume, and cell body area of astrocytes in subiculum (Y axes represent cell count density in µm²). 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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A. CA1
B. CA2/3
C. CA4

D. Dentate gyrus
E. Subiculum
F. Entorhinal cortex

G. Entorhinal subcortical WM
H. Temporal cortex
I. Temporal subcortical WM

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