U.PORTO

Mestrado Integrado em Medicina

Dissertação | Artigo de Investigação

Neuroinflammation patterns in Alzheimer´s disease, Dementia with Lewy bodies and cases with mixed pathology

António Gil Cabral de Azevedo

M

2022

Neuroinflammation patterns in Alzheimer´s disease, Dementia with Lewy bodies and cases with mixed pathology

Padrões da neuroinflamação na Doença de Alzheimer, Demência de Corpos de Lewy e casos de patologia mista

---

Dissertação de candidatura ao grau de Mestre em Medicina

Instituto de Ciências Biomédicas Abel Salazar

Universidade do Porto

Estudante

Nome completo:	António Gil Cabral de Azevedo
Contactos:	antoniogcazevedo@gmail.com 00351962032476

Orientador

Nome completo:	Ricardo Jorge Ferreira Taipa
Grau académico:	Doutorado
Título profissional:	Médico Assistente Hospitalar de Neurologia - Unidade de Neuropatologia e Serviço de Neurologia do Departamento de Neurociências; Prof. Auxiliar Convidado da Unidade de Anatomia e Fisiologia do Sistema Nervoso do ICBAS/UP

Junho de 2022

Neuroinflammation patterns in Alzheimer´s disease, Dementia with Lewy bodies and cases with mixed pathology

Padrões da neuroinflamação na Doença de Alzheimer, Demência de Corpos de Lewy e casos de patologia mista

Dissertação de candidatura ao grau de Mestre em Medicina
Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto

Estudante

António Gil Cabral Azevedo

(António Gil Cabral de Azevedo)

Orientador

(Professor Doutor Ricardo Jorge Ferreira Taipa)

Porto, Junho de 2022

Dedicatórias

Aos Professores e Orientadores que marcaram o meu percurso escolar e académico até ao momento presente. Estou-lhes muito grato e sei que lhes devo muito de quem sou.

À minha família e amigos, por todo o apoio ao longo do meu percurso.

À memória do meu avô, Domingos Dias de Azevedo.

Agradecimentos

Ao meu orientador, Professor Doutor Ricardo Taipa, por toda a disponibilidade, apoio e críticas construtivas ao longo deste projeto, desde o seu desenho e candidatura a financiamento até à discussão de resultados e revisão da tese. A extensa experiência clínica e científica do Professor, bem como as suas ímpares capacidades humanas tornaram este projeto não só realizador como também verdadeiramente agradável e enriquecedor.

À Dra. Inês Reis e à equipa do Banco de Cérebros Português, que recolheram e processaram as amostras dos dadores, sem a qual este projeto não teria sido possível. Estimo muito todo o apoio e amizade ao longo do projeto.

À Dra. Catarina Damas pelo apoio com a colheita de dados clínicos.

Ao grupo de investigação da Professora Doutora Mariana P. Monteiro da Unidade Multidisciplinar de Investigação Biomédica (UMIB), e em especial à Professora Doutora Sofia S. Pereira que foi uma verdadeira co-orientadora não oficial neste projeto. Para além de contribuírem com apoio técnico e científico ao nível da digitalização de lâminas e análise de imagem, deram igualmente contributos decisivos para ultrapassar vários desafios logísticos, burocráticos e técnicos que poderiam ter inviabilizado a realização desta tese. Destaque também para a Dra. Madalena M. Costa por todo o apoio técnico e amizade. Estou muito grato a toda a equipa UMIB por todo o apoio, excelente dinâmica de trabalho e amizade.

À Fundação Professor Ernesto Morais (FPEM) e à memória do notável e inspirador Professor Ernesto Morais. Este estudo seria inteiramente impossível sem o financiamento da FPEM, a quem estou muito grato por me propiciarem as condições para poder dar um maior contributo para a ciência e sociedade neste projeto e ao longo da minha futura carreira clínica e científica.

Ao Professor Doutor Pedro Oliveira, pelo seu apoio com a análise estatística dos dados.

Ao IPATIMUP, na pessoa do Dr. João Vale e da Dra. Mónica Curado, pelo serviço de digitalização de lâminas, realizado sempre com grande rapidez, profissionalismo e simpatia.

Aos doentes e familiares afetados pela demência que são a principal inspiração para o trabalho. Em particular aos dadores que participaram com amostras para este projeto, cujo contributo é inestimável. Que a esperança num futuro melhor para os afetados presentes e futuros nunca seja esquecida.

This page is intentionally left blank

Resumo

Enquadramento e objetivos: A Doença de Alzheimer (AD, sigla inglesa) e a Demência com Corpos de Lewy (DLB, sigla inglesa) são as duas causas mais comuns de demência neurodegenerativa. Estudos de neuropatologia indicam que mais de 60% dos doentes com AD e DLB apresentam co-patologia na autopsia, que está associada a fenótipos mais severos. Este trabalho procura caracterizar a neuroinflamação em doentes com formas puras e sobrepostas de neuropatologia associada a AD e DLB, contribuindo para o desenvolvimento de novos biomarcadores para futuras terapêuticas mais individualizadas e ultimamente melhorar os resultados em saúde e o bem-estar dos doentes com demência.

Métodos: A neuroinflamação em substância cinzenta e branca de córtex frontal humano *post mortem* foi caracterizada em termos de ativação microglial através imunohistoquímica para CD68 em casos neuropatologicamente definidos de AD, Doença de Corpos de Lewy (LBD, sigla inglesa; que corresponde a DLB ou Demência associada à Doença de Parkinson) e de casos de co-patologia. Um protocolo de neuropatologia digital para quantificação semiautomática em imagens de slide inteiro foi estabelecido, otimizado e validado.

Resultados: Dezoito casos foram selecionados (valores expressos com média [desvio padrão]): AD (n=6; idade ao inicio de sintomas [AASO, sigla inglesa], 62.7 [7.5] anos; idade à data da morte [AAD, sigla inglesa], 69.8 [5.9] anos), AD/LBD (n=4; AASO, 55.3 [3.1] anos; AAD, 64.0 [6.5] anos), LBD (n=5; AASO, 62.8 [9.0] anos; AAD, 72.8 [7.6] anos), Controlo (n=3; AAD, 60.3 [12.0] anos). Verificou-se um aumento de carga proteica de CD68 na substância cinzenta do giro frontal médio (MFG, sigla inglesa) em casos de AD e de co-patologia relativamente aos casos controlo e de Doença de Corpos de Lewy (LBD, sigla inglesa). Não foram verificadas diferenças estatisticamente significativas entre casos AD e casos de co-patologia. Os casos de LBD não diferiram dos casos controlo. Não foram encontradas diferenças entre os grupos de estudo na substância branca do MFG.

Conclusões: Verificou-se que os casos com co-patologia tinham uma carga proteica de CD68 sugestiva de ativação microglial na substância cinzenta do MFG, que se assemelhava mais aos casos de AD pura que aos casos de LBD pura. Os resultados atuais apoiam uma influência prevalente da patologia de AD nos casos de co-patologia em termos de neuroinflamação, em contexto *post mortem* de doença avançada. Os casos de AD pura e de co-patologia não diferiram para esta característica o que poderá sugerir a ausência de efeito aditivo entre patologia de AD e LBD em termos da neuroinflamação neste contexto. Será necessário um maior tamanho de amostra e considerar outros marcadores de neuroinflamação de forma a poder inferir se existe um efeito aditivo das patologias de AD e LBD em casos de co-patologia em termos de neuroinflamação. Será

importante a caracterização detalhada de correlações clínico-patológicas em casos de co-patologia AD/LBD de forma a informar atuais e futuros ensaios clínicos dirigidos à neuroinflamação em AD e LBD.

Palavras-chave: Demência, Doença de Alzheimer, Demência de Corpos de Lewy, Neuropatologia, Neuroinflamação, co-patologia

Abstract

Background and aims: Alzheimer's Disease (AD) and Dementia with Lewy Bodies (DLB) are the two most common causes of neurodegenerative dementia. Neuropathology studies indicate that more than 60% of AD and DLB patients present co-pathology at autopsy, which is associated with worse phenotypes. This work aims to characterize neuroinflammation in patients with pure and overlapping Alzheimer and Lewy Body pathology, to contribute to the development of new biomarkers, more tailored future therapeutic approaches, and ultimately better outcomes and healthcare for patients with dementia.

Methods: Neuroinflammation in human *post mortem* - frontal cortex gray and white matter was characterized in terms of microglial activation by CD68 immunohistochemistry in neuropathologically defined cases of AD, Lewy Body Disease (either DLB or Parkinson's Disease Dementia) and co-pathology cases. A semi-automatic whole slide image digital neuropathology pipeline was established, optimized and validated.

Results: Eighteen cases were selected (values expressed as mean [standard deviation]): AD (n=6; age at symptom onset (AASO), 62.7 [7.5] years; age at death (AAD), 69.8 [5.9] years), AD/LBD (n=4; AASO, 55.3 [3.1] years; AAD, 64.0 [6.5] years), LBD (n=5; AAOS, 62.8 [9.0] years; AAD, 72.8 [7.6] years), Control (n=3; AAD, 60.3 [12.0] years). Cases with AD and co-pathology cases were found to have increased CD68 protein load in the middle frontal gyrus (MFG) gray matter relative to control and Lewy body disease (LBD). No statistically significant difference was found between AD and co-pathology cases. LBD cases did not to differ from controls. No differences were found between study groups in MFG white matter.

Conclusions: Co-pathology cases were found to have a CD68 protein load suggestive of microglial activation in MFG gray matter that more closely resembled pure AD cases than pure LBD cases. Current results support a prevailing influence of AD pathology in co-pathology cases in terms of neuroinflammation at *post mortem* late stage disease. Pure AD cases and co-pathology cases did not differ for this feature which could suggest an absence of additive effect between AD and LBD pathology in terms of neuroinflammation in this context. A bigger sample size and considering other neuroinflammation markers will be required in order to infer if there is an additive effect of AD and LBD pathology in co-pathology cases in terms of neuroinflammation. A detailed characterization of clinicopathological correlations in cases with AD/LBD co-pathology will be important in order to inform current and future clinical trials targeting neuroinflammation in AD and LBD.

Keywords: Dementia, Alzheimer's Disease, Lewy Body Dementia, Neuropathology, Neuroinflammation, co-pathology

Abbreviations

αSYN - alpha-synuclein

^{18}F-FBB - ^{18}F-florbetaben

Aβ – amyloid beta

AD – Alzheimer's Disease

ADNC – Alzheimer's Disease Neuropathological Change

CAA – Cerebral Amyloid Angiopathy

CD68 – Cluster of Differentiation 68

DAB – 3,3'-Diaminobenzidine

DAT – Dopamine Transporter

DLB – Dementia with Lewy Bodies

EOAD – Early Onset Alzheimer's Disease

ER - Endoplasmic Reticulum

ERAD - Endoplasmic reticulum associated degradation

FDA – United States of America Food and Drug Administration

FTD – Frontotemporal Dementia

GCase - glucocerebrosidase enzyme

GD - Gaucher's disease

GWAS – Genome Wide Association Study

HLA-DR – Human Leukocyte Antigen - DR isotype

Iba1 – Ionized calcium-binding adaptor molecule

iLBD – incidental Lewy Body Disease

iPSC – Induced Pluripotent Stem Cells

LB – Lewy Bodies

LBD – Lewy Body Disease/Disorder

LBP – Lewy Body Pathology

LN – Lewy Neurites

LOAD – Late Onset Alzheimer's Disease

MCI – Mild Cognitive Impairment

MFG – Middle Frontal Gyrus

MHC-II – Major Histocompatibility Complex class 2

MMSE – Mini-Mental State Examination

NFT – Neurofibrillary Tangles

PAM - Proportion of morphologically active microglia

PDD – Parkinson's Disease Dementia

PET – Positron Emission Tomography

REM sleep – Rapid Eye Movement sleep

RGB – Red Green Blue

ROI – Region of Interest

scRNA-seq – Single cell RNA sequencing

TIFF - Tag Image File Format

WSI – Whole Slide Image

Index

Background 15
AD neuropathological change and Lewy body pathology in AD and DLB patients 16
Neuroinflammation implicated in neurodegeneration 17
Neuroinflammation in AD 17
Neuroinflammation in DLB 20
Neuroinflammation based therapies under development 24
Methods 26
Study design and data 26
Participants 26
Clinical Information 26
Immunohistochemistry 27
Slide digitization 27
Quantification of immunohistochemistry-stained area 28
Statistical analysis 29
Ethics review 30
Results 31
Socio-demographic and clinical characterization 31
Characterization of middle frontal gyrus CD68 protein load by immunohistochemistry 31
Gray matter 31
White matter 32
Discussion 33
Conclusions 45
Disclosure Statement 46
Tables and Figures 47
References 61

Table List

Table I – Demographic and clinical features of the individuals assessed 53
Table II – Area of selected gray and white matter and regions of interest 54

Figure List

Figure 1 - Region of interest selection within the middle frontal cortex on a representative whole slide image on QuPath..... 47
Figure 2 – Representative image of white and gray matter segmentation during ROI selection on QuPath..... 48
Figure 3 – Example of a region of white matter within a whole slide image of middle frontal cortex exported from QuPath (A) and imported into ImageJ in smaller images (B to E; amenable to image processing with the available computing power)..... 49
Figure 4 – Semi-automated selection of tissue area through thresholding..... 50
Figure 5 – Semi-automated selection of 3,3'-Diaminobenzidine (DAB) stained area through color deconvolution and thresholding..... 51
Figure 6 – Comparison of pictures of 3,3'-Diaminobenzidine (DAB) stained area selected using different intensity thresholds in representative white and gray matter images of a case with AD. Ratio of DAB stained area quantified by the particle analysis ImageJ plugin are plotted on the bottom right of each picture. 52
Figure 7 - Representative middle frontal gyrus gray matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 50 µm. 55
Figure 8 - Representative middle frontal gyrus gray matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 20 µm. 56
Figure 9 – Middle frontal gyrus gray matter CD68 protein load assessed through the ratio of tissue area positive for CD68 in immunohistochemistry staining of *post mortem* human samples. Patient groups compared included: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease; n=6), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease; n=4), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease; n=5), control (individuals without clinical dementia at age of death; n=3). Results of each of the groups for percentage of CD68 tissue staining are plotted in a boxplot representation. Asterisks indicate statistical significance (one asterisk corresponds to $P \leqslant 0.05$; two asterisks to $P \leqslant 0.01$; three asterisks to $P \leqslant 0.001$) and ns indicates no statistically significant differences were found ($P > 0.05$). 57
Figure 10 - Representative middle frontal gyrus white matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 50 µm. 58

Figure 11 - Representative middle frontal gyrus white matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 20 μm. 59
Figure 12 - Middle frontal gyrus white matter CD68 protein load assessed through the ratio of tissue area positive for CD68 in immunohistochemistry staining of *post mortem* human samples. Patient groups compared included: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease; n=6), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease; n=4), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease; n=5), control (individuals without clinical dementia at age of death; n=3). Results of each of the groups for percentage of CD68 tissue staining are plotted in a boxplot representation. No statistically significant differences were found between groups (ANOVA, p=0.2675). 60

Background

Alzheimer's Disease (AD) and Dementia with Lewy Bodies (DLB) are the two most common causes of neurodegenerative dementia in the elderly, causing a substantial impact in the lives of patients, caretakers and society as a whole [1–3] These pathologies have a rapidly growing prevalence that is associated with population ageing (50 million patients suffering from dementia globally in 2015 and up to 152 million estimated in 2050)[1,2]. AD and DLB are characterized by deposition of specific abnormal protein aggregates that are associated with neuronal death and disfunction as well as with inflammatory activity and lesions [3–6]. Cumulative data in the scientific literature suggests an important role of neuroinflammation in AD and DLB pathogenesis.[5–8]

Furthermore, neuropathology studies indicate that simultaneous presentation of multiple neurodegenerative pathologies in human *post mortem* samples are common (e.g. presence of AD pathology in up to 80% of DLB patients and presence of alpha-synucleinopathy in up to 60% of AD patients [9,10]). Individuals with multiple neuropathologies at autopsy are more likely to have a diagnosis of dementia [11] and are also associated with worse phenotypes. [9,10] However, the impact of the presence of multiple simultaneous neuropathologies in the modulation of neuroinflammation in different brain areas is still poorly characterized.[12]

A deeper understanding of the biology of neuroinflammation in patients with multiple neurodegenerative diseases could potentially shed new insight on the substantial clinical heterogeneity of these diseases. It could thus help promote the development of new biomarkers and better tailored therapeutic approaches, so as to deliver better outcomes and healthcare to patients with dementia.

Recent developments make the need for a deeper insight on the biology of neuroinflammation in patients with multiple neurodegenerative diseases even more pressing. A rising number of novel therapeutic strategies targeting neuroinflammation pathways highlighted by human genetic studies are currently under development.[13] If these promising new therapies show efficacy in clinical trials it will be important to understand implications for the substantial number of patients with overlapping neuropathologies. A better understanding of the underlying biology might contribute to the development of biomarkers that may help guide future therapeutic decisions for these patients.

AD neuropathological change and Lewy body pathology in AD and DLB patients

Alzheimer's disease (AD) is the most common neurodegenerative cause of dementia, accounting for 60 to 80% of all dementia cases. [3,14,15] AD is characterized by deterioration of cognitive functions, including memory, impairment of daily living activities, and behavioural and psychological symptoms.[16] AD neuropathological change (ADNC) is characterized by two major neuropathological hallmarks: senile plaques, mainly composed of extracellular deposits of amyloid beta (Aβ); and neurofibrillary tangles (NFT), consisting of intracellular aggregates of aberrantly phosphorylated tau protein.[3,8] This is accompanied by neuronal and synaptic loss, dendritic and axonal changes and neuro-inflammation.[3,8,15]

Dementia with Lewy Bodies (DLB) is the second most common cause of dementia, accounting from 4.2% up to 30% of dementia cases.[5,14] Clinical features of DLB include fluctuating cognition, visual hallucinations, rapid eye movement (REM) sleep behavior disorder and parkinsonism [5,17,18] Patients with DLB are found on neuropathology to have Lewy body pathology (LBP) with Lewy bodies (LB) and Lewy neurites (LN), formed primarily of alpha-synuclein (αSYN).[5]

Lewy body deposition is a hallmark not just of DLB but also of Parkinson's Disease (PD). [19,20] When PD develops first and dementia only develops after 1 year or more, a diagnosis of Parkinson's Disease Dementia (PDD) is made.[3,20] This differentiation between DLB and PDD is not consensual in the literature owing to absence of definite pathological criteria to discriminate them.[3] PD/PDD and DLB are therefore described in some literature as Lewy Body Disorders (LBD)[21–23]. Some reported differences between PDD and DLB pathology are a higher amount of Aβ and tau load in DLB[20,23–25], and a more severe α-SYN load in the amygdala in DLB, as well as a higher deposition of α-SYN in the claustrum (increasing load from PD without dementia to PDD and from PDD to DLB)[26]. Another recently reported difference is higher cerebral amyloid angiopathy (CAA) in DLB relative to PDD[27]. The difference in CAA scores was reported to be more significant in the parietal and occipital lobes.[27] DLB is also reported to have a more rapid disease progression than PDD.[24,27]

AD and DLB have significant clinical overlap with some DLB patients having early prominent memory impairment[10] and some late-stage AD patients developing parkinsonism and visual hallucinations.[10,28] Overlapping AD neuropathological change (ADNC) and Lewy body pathology (LBP) are also very common in neuropathology findings of AD and DLB patients. According to the literature up to 60% of sporadic AD patients exhibit LBP and among DLB patients up to 66% have amyloid plaques and 10% have NFTs. [10] In some studies up to 80% of DLB patients are found to have ADNC.[9] Patients with coexisting ADNC and LBP are described to have worse phenotypes[9,10,29,30], namely an earlier age of symptom onset.[10,29,30] Shorter survival after onset was found to be

associated with severe LBP in a cohort of AD, AD/DLB and DLB cases[10], but not in a bigger cohort looking at AD with and without LBP[30]. A more pronounced cerebral atrophy is associated with severe ADNC.[10] Presence of autonomic dysfunction is reported to correlate strongly with the presence of alpha-synucleinopathy, which may help identify patients with mixed AD and LBP in the clinical setting.[10] Other reported features associated with AD patients with LBP pathology relative to pure AD are a higher frequency of at least one APOEe4 allele and higher scores in the Neuropsychiatric Inventory Questionaire and in the Unified Parkinson Disease Rating Scale motor Score.[30] DLB patients with higher Aβ deposition *in vivo*, assessed by ^{18}F-florbetaben (^{18}F-FBB) PET, were also found to have earlier onset of symptoms, higher cognitive and neuropsychiatric burden, as well as decreased dopamine transporter (DAT) activity.[29] DLB patients with higher LBP pathology in *post mortem* studies were reported to have a shorter disease duration.[31]

Individuals with multiple neuropathologies at autopsy are reported to be more likely to have a diagnosis of dementia among community dwelling older persons.[11]

Neuroinflammation implicated in neurodegeneration

Cumulative data from genetic[32,33], transcriptomic [34] and *post-mortem* pathology[4,5,8,35,36] in humans suggests an important role of neuroinflammation and glial cells (in particular microglia) in neurodegenerative disorders. Recent scientific literature implicates microglia, complement, and other immune-related pathways as early mediators of synaptic dysfunction in the absence of plaques and overt inflammation [13,37]

Neuroinflammation in AD

Sporadic AD has a high level of heritability (approximately 70%). [32] Over the last 10 years, high-throughput genomic approaches such as genome wide association studies (GWAS) and sequencing studies enabled the discovery of more than 40 genes/loci linked to the AD risk.[32] A recent international GWAS meta-analysis looking at AD and proxy-AD (probable AD based on questionnaire) cases versus controls found a total of 75 loci linked to risk of AD and AD related dementias, expanding the understanding of their genetic architecture.[33] Pathway analysis supported as the most significant gene sets associated with AD: amyloid and tau, lipids, endocytosis and immunity (namely macrophage and microglial activation).[33] Linked loci, pathway analysis and cell-type association of findings suggest an important role for microglia in AD pathology. [32,33] Some analysis of the genetic data indicate in addition that many of the functional variants uncovered mainly affect gene expression of myeloid cells and thus potentially that of microglia.[32] Some AD risk

variants may also be located in microglial-specific enhancers.[32] Reports of non-synonymous variants in TREM2, associated with AD risk in particular led to relevant hypothesis-driven studies in microglia. TREM2 has been proposed to be protective in AD pathology since the AD-associated mutations (R47H, R62H) likely impair its physiological functions in terms of phagocytosis and clearance of Aβ peptides as well as in the compaction of amyloid plaques and barrier formation.[13,32]

Amyloid beta, hyperphosphorylated tau and microglia activation are reported to sequentially affect different brain regions in given spatial and temporal patterns in AD.[38–43] Data from *in vivo* imaging studies, using PET TSPO binding in particular linked AD and microglial activity, suggesting that activated microglia burden correlates with cognitive decline in AD.[44,45] Microglia burden is reported to correlate strongly with amyloid pathology in the early MCI stage and to follow a similar pattern to amyloid deposition in the time sequence of affected brain regions.[45] Amyloid deposition is described to first be detected throughout the neocortex and then to expand ventrally to the allocortex, midbrain, brainstem and eventually cerebellar areas.[40,45] In MCI patients, microglia burden seems to be increased in cortical regions that are associated with early amyloid deposition (for instance frontal, occipital, parietal and temporal cortical regions) and to spare brain regions only affected by amyloid deposition in later stages of AD (for instance striatum and cerebellum).[45] In later AD stages, these studies report that microglial burden is more closely associated with tau pathology, with a particularly strong neuroinflammation signal in temporal regions, also strongly associated with tau pathology.[45] Recent studies concerning the role neuroinflammation and microglia in AD pathology point to a complex biology in which microglia cells in different states can have either deleterious or protective effects in different stages of AD pathology.[13,41] The literature, namely from *in vivo* TSPO PET studies of neuroinflammation in patients, seems to suggest an initial protective peak in microglial response at the early or asymptomatic stages of AD, possibly associated with initial amyloid beta deposition, followed by a late deleterious pro-inflammatory peak in late stages of AD, possibly associated with later tau spreading.[41,45,46]

In *post mortem* studies AD brain samples were shown relative to controls to have a greater microglial activation[47] with greater microglia density and number[4,6,8,36] mostly around amyloid plaques but also apparently unassociated with plaques[8]; and greater astrogliosis, mostly associated with plaques but also found apparently unassociated with plaques[8]. These neuroinflammatory pathological markers in late stage AD human tissue were found to have a similar pattern in both early age of onset AD (EOAD) and late age of onset AD (LOAD), though the severity of the pathological markers in the younger group was higher.[8] The proportion of morphologically active microglia (PAM) is reported to have a stronger association with AD status than microglia density alone, having an effect size comparable to that of APOE ε4, the strongest genetic risk factor for

Alzheimer's disease.[35] Microglia subpopulations with different immunophenotypes in neuropathology were also show to correlate differently with ADNC and clinical features such as dementia status and cognition score.[36] In particular, cluster of differentiation (CD)68 and ionized calcium-binding adaptor molecule (Iba1) were reported to have a non-significantly associated expression pattern in microglia and correlate differently with ADNC features and clinical features.[36]

CD68 is a lysosomal and endosomal transmembrane glycoprotein associated with phagocytic activity [5,36,48]. As a lysosomal marker it is mainly expressed in the microglial soma[48], being also expressed in other macrophages such as neutrophils and monocytes.[48] CD68 protein load is also reported to increase with normal ageing possibly associated with aging-dependent lysosomal accumulations of lipofuscin within senescent microglial cells. [49]

CD68 protein load in human *postmortem* neuropathology studies was found to be associated with clinical dementia status in AD, poor cognitive function and ADNC pathology (namely neuritic plaques and tangles)[4,6,8,36] CD68 is also associated in human single cell sequencing (scRNA-seq) studies with transcription signatures associated with mild cognitive impairment and AD dementia[50]

Iba1 is a cytoplasmatic protein constitutively expressed by microglia[36] (also expressed in peripheral macrophages[48]) that is upregulated in inflammation[36,48]. It is associated with cytoskeletal reorganization and microglia motility and migration.[36,48] In human neuropathology studies, contrary to CD68, Iba1 expression was associated with absence of dementia and better cognition scores[36]

Interestingly, APOE genotype is reported to influence microglia such that the protective allele ε2 is associated with a protective microglia profile (increased Iba1 expression and decreased CD68), while the detrimental APOE ε4 is associated with a detrimental microglia profile (increased CD68 expression).[36] In accordance with the literature, this implies that microglia dynamics could partially mediate APOE genotype effect in AD.[51]

Neocortex regions such as the middle frontal gyrus, being implicated in AD neuroinflammation, yet relatively more preserved than temporal regions in terms of tau pathology in late AD[39], could be particularly promising to explore the neuroinflammation dynamics of AD, as shown in previous studies.[36]

Neuroinflammation in DLB

While the role of neuroinflammation in AD pathology is well established, its role in DLB is less well characterized.[5] DLB has an estimated heritability of 60%.[14] Though better powered genetic studies are needed, there are 3 established genetic risk factors consistent across 2 recent GWAS studies of DLB: SNCA, GBA, and APOE. [14] These 3 established genetic risk factors were again implicated in an even more recent genome sequencing study that also implicated BIN1 and TMEM175.[52]

While SNCA, GBA and TMEM175 are shared risk factors with Parkinson's Disease (PD), APOE and BIN1 are a shared common risk with AD.[33,52,53] There is debate among the scientific community regarding APOE and DLB risk.[14,51,54] In some studies APOE was reported to contribute directly to risk of DLB even in the absence of significant AD neuropathological change (ADNC).[14,51] In other studies the increase in DLB risk due to APOE was linked to presence and severity of ADNC[14,51], and ADNC is known to lower symptom age of onset in DLB. [10,14,29] A recent study indicates that APOE is associated with DLB with AD co-pathology but not with pure DLB.[54] In contrast, a different study suggests that APOE ε4 is associated with increased α-SYN seeding activity in AD patients with LBP as well as in DLB with minimal ADNC.[55]

APOE and SORL1 (that is implicated in AD and in some DLB genetic studies[13,14]) are highly expressed in microglia suggesting their impact on risk of neurodegenerative disease could be microglia associated.[13] These genes are associated with lysosomal activity and lipid metabolism, which are implicated in the literature as having a role in the regulation of microglia functional states.[13] AD and DLB implicated BIN1 is also associated with the endolysosomal network.[56] Although BIN1 expression is present in several cell types, the GWAS implicated variants are located in a regulatory region downstream of BIN1, predicted to be a microglia-specific enhancer.[56] Deletion of this implicated enhancer region decreased BIN1 expression in induced pluripotent stem cells (iPSC)-derived microglia but not in neurons and astrocytes, further suggesting BIN1 GWAS implicated variants could be acting through microglia[56]

GBA encodes the lysosomal enzyme glucocerebrosidase (GCase) and is also implicated in Gaucher's disease (GD) and Parkinson's Disease.[57] Biallelic (homozygous or compound heterozygous) mutations in GBA can cause Gaucher's disease in which glucosylceramide accumulates in visceral organs and, in a minority of cases, the central nervous system (neuronopathic GD).[58] The literature suggests possible mechanisms for GBA-associated neurodegeneration in alpha-synucleinopathies. Glucosylceramide, may lead to α-synuclein accumulation, and inversely, α-synuclein accumulation may lead to reduced GCase activity encoded by the GBA gene, forming a vicious pathological cycle.[57]

Another possible mechanism is endoplasmatic reticulum (ER) associated degradation (ERAD) impairment and ER stress-related cell death which have also been implicated in alpha-synucleopathies.[57] GBA mutation carriers have a 10%–30% chance of developing PD and PD patients carrying a GBA mutation have an earlier disease onset and a higher risk of dementia.[58] A recent study has shown evidence of *in vivo* presence of brain microglial activation in GBA mutation carriers without either a prodromal or clinical diagnosis of PD in brain regions susceptible to Lewy body formation through PET (using ^{11}C-(R)-PK11195 BP_{ND} PET). In particular ^{11}C-(R)-PK11195 suggested increased microglial activity relative to controls in substantia nigra, bilaterally in the occipital and temporal cortices, cerebellum, left hippocampus, and central and anterior mesencephalon. [58] Though it is not possible to discern if this microglia activation is a cytotoxic or neuroprotective process and further validation studies will be important, this study suggests GBA associated risk of alpha-synucleinopathy could be in part due to its role in modulating neuroinflammation. The literature indicates a higher rate of GBA mutations in AD/DLB (odds ratio of 4.6 relative to control) and particularly pure DLB (odds ratio of 7.6 relative to control) but no difference in GBA mutation rate in AD.[59] According to a recent study, GBA is associated with pure DLB but not with DLB with AD co-pathology.[54]

LBD and PD implicated TMEM175, like GBA, is equally implicated in lysosomal function and α-synuclein aggregation[60,61]

Of note, in a large whole exome sequencing study of more than 1000 DLB patients, mutations in genes known to cause other neurodegenerative disorders such as AD, PD or frontotemporal dementia (FTD) were very rare, suggesting a limited role for the known genetic modifiers of these disorders in DLB and that yet undetermined genetic loci may play a role in the genetic component of DLB.[62]

Alpha-synuclein is reported to sequentially affect different brain regions in given spatial and temporal patterns in DLB and PD/PDD.[18,21,25,39,42] *In vivo* microglia activation as measured by TSPO PET imaging using ^{11}C-PK11195 binding seems to indicate, in patients assessed within a year of symptom onset, an early microglial activation in the putamen and substantia nigra in DLB and PD, and additionally for DLB also in the caudate, several cortical regions and even the cerebellum.[63] Another study looking at DLB patients with approximately 4 years of disease duration (average of 4.2 years with 2.7 years of standard deviation), also compared patients with different severity of cognitive impairment (mild versus moderate/severe cognitive impairment, respectively above and below a score of 65 in the Addenbrooke's Cognitive Examination-Revised [ACE-R])[64]. In this study, overall, DLB with mild cognitive impairment are reported to have higher ^{11}C-PK11195 binding

associated with higher microglial activation and moderate/severe cases to have lower ^{11}C-PK11195 binding associated with a lower microglial activation, below that of age matched controls.[64] Of the regions assessed, there was a significantly higher ^{11}C-PK11195 binding in mild cases in the inferior and middle temporal gyrus, the fusiform gyrus, the putamen, the inferior frontal gyrus and the cuneus. Only the caudate was significantly lower in ^{11}C-PK11195 binding relative to controls in moderate/severe cases.[64] The regions most strongly correlated with ACE-R score were the caudate and cuneus.[64] These results suggest a non-linear spectrum of microglial elevation, with an initial high microglia activation that decreases in later disease stages.[12,64]

A TSPO PET study of PD and PDD found an increased neuroinflammation in PD relative to control and in PDD relative to PD, as well as an inverse correlation of neuroinflammation in temporo-parietal, frontal and occipital cortex and MMSE score.[65] Maximal microglial activation was found in the parieto-occipital cortex, with other affected areas comprising frontal, temporal and cingulate cortex and in PDD also the brain stem[65] These findings seem to point to an early increase of neuroinflammation in PD/PDD, starting before onset of dementia, that then persists during dementia.[65] A more recent meta-analysis with data from PET studies using second generation TSPO ligands suggests that in PD there is a more localized neuroinflammation mainly affecting the midbrain.[66]

The interplay in DLB and PD/PDD between α-SYN deposition, ADNC and microglial activation is considered in the literature to be a particularly unexplored and potentially important area of further research, as ADNC, implicated in microglial activation in AD, is also present in many patients that have Lewy Body Disorders with AD co-pathology.[12]

A recent *post mortem* neuropathology study showed absence of prominent microglial activation and highlighted an increased T-lymphocyte recruitment in DLB patient samples of the middle temporal gyrus relative to controls.[5] A different *post mortem* neuropathology study focused on hippocampal pathology suggested a low microglia density in DLB coupled with an increased proportion of dystrophic microglia (with processes that are spheroidal, beaded, de-ramified, or fragmented) to all microglia.[6] While neuroinflammation is implicated in genetic and transcriptomic studies across neuropathologies including DLB[34], the biology of the neuroinflammation may differ between diseases and brain regions, with a possible stronger role for T-lymphocytes than microglia in DLB associated neuroinflammation in the middle temporal gyrus at the late disease stage represented in *post mortem* studies.[12]

Still within Lewy Body Disorders, PD and PDD *post mortem* neuropathology studies also point to a role of neuroinflammation[67]. Increased microglial activity assessed by human leucocyte antigen –

isotope DR (HLA-DR) was reported in the amygdala in PDD[68] and in the hippocampus, transentorhinal, cingulate and temporal cortex in PD/PDD[69] in *post mortem* studies, however HLA-DR at *post mortem* was not reported to correlate with clinical phenotype[68] In a *post mortem* study with PD/PDD patients looking at CD68 and major histocompatibility complex class 2 (MHC-II) markers in the substantia nigra, only CD68, associated with phagocytic activity, was found to correlate with clinical features namely disease duration.[70] In this cohort increased CD68 correlated with shorter disease duration, being more expressed in PD/PDD patients with a disease duration shorter than 10 years[70]. As in this cohort age of symptom onset correlated negatively with disease duration[70], this result could suggest either that CD68 associates with faster disease progression or to longer asymptomatic stages. Failure of MCH-II markers to correlate with clinical features in PDD *post mortem* studies in contrast to CD68 is suggestive that, as in AD pathology, in PD/PDD different microglial markers may have different correlations and relevance to clinical features.

In a different *post mortem* study looking at incidental LBD (iLBD; Braak PD 1-3) and PD/PDD study, CD68 expressing amoeboid (morphologicaly activated) microglia were found to increase in iLBD relative to control and in PD/PDD relative to iLBD in the substantia nigra.[71] CD68 expressing amoeboid microglia were also found to be increased in PD/PDD relative to control and iLBD in the hippocampus, particularly in CA2 and CA3.[71] Iba1 expressing microglia were found in this study to be in an increasing proportion in morphologically more activated forms in the substantia nigra and CA2 from control to iLBD and from iLBD to PD/PDD.[71] TLR2 expressing microglia were found to be increased in iLBD relative to controls in the substantia nigra and the hippocampus and to have a more reduced expression in PD/PDD in the substantia nigra (still above control levels) and in the hippocampus (equivalente to control levels).[71] TLR2 was also found to co-localize with morphologically activated Iba1 microglia in the substantia nigra and hippocampus and in amoeboid CD68 expressing microglia in the substantia nigra.[71] Of note, this study indicates that microglial activation in iLBD/PD/PDD pathology seems to correlate with alpha-synuclein deposition stages in terms of time sequence and affected brain regions. It also suggests microglial activation precedes neuron loss in the substantia nigra and clinical symptoms, as CD68 amoeboid microglia were found in the substantia nigra in iLBD, in a context where neuron loss was not yet considered to be significant. It additionally indicates a non-linear profile of increase in different types of microglia markers in different brain regions.

Other findings in PD/PDD *post mortem* studies were CD4+ T-cell infiltration (amygdala and substantia nigra in PD and PDD[68]). CD4+ T cell counts in the amygdala are described to correlate with activated microglia, α-synuclein and tau pathology.[68] Astrogliosis in the substantia nigra was described in patients with PD (statistically significant difference from controls in PD without

dementia but not in PDD)[68]. A recent single nuclei RNA sequencing study of idiopathic PD *post mortem* midbrain indicated that both neurons and glia are enriched in expression of GWAS implicated genes and that there is "pan-glial" activation.[72] This study reported an increase in nigral microglia and microglial pro-inflammatory shift with increased expression of IL1B, GPNMB and HSP90AA1.[72] It also indicated reduced oligodendrocyte numbers and their upregulation of stress induced S100B as well as CD44 overexpression in astrocytes.[72]

While the literature seems to indicate in *post mortem* studies a higher microglia activity in PD/PDD and lower in LBD, both seem to share a stronger role of T-lymphocytes in their pathology relative to what is described in AD pathology.[12,73]

Neocortex regions such as the middle frontal gyrus, being implicated in LBP neuroinflammation[63,64], yet relatively more preserved than temporal regions or the substantia nigra[12], could be particularly promising to explore the neuroinflammation dynamics of LBP.

Neuroinflammation based therapies under development

AD therapeutic strategies targeting amyloid beta and the amyloid cascade, despite successfully reducing amyloid deposition in human clinical trials as confirmed by PET through different methods, are considered in some literature to not yet have been fully translated to effective treatment options for patients.[74,75] Only one disease-modifying monoclonal antibody, Aducanumab, has been recently approved by the United States Food and Drug Administration (FDA).[75] In some literature there is substantial controversy regarding the approval process of Aducamumab, namely concerning failure to meet clinical endpoints in clinical trials, cost-effectiveness, potential adverse effects and need for regular monitoring due to safety issues.[75,76]

Risk factor genes and pathways from genome wide associations studies that are associated with microglia and neuroinflammation are being studied as new potential targets for therapeutic strategies for AD.[13,74] Modulation of the neuroimmune system, for instance through the TREM2 pathway and microglia function, is emerging as an innovative new therapeutic strategy.[13,74] In comparison to AD, there is a reduced number of DLB therapies in clinical trials and in development pipelines and many of these were initially developed for AD or PDD.[77]

The future success of neuroinflammation targeting therapeutic strategies in AD and other neuropathologies such as DLB will require a deeper insight of the underlying biology in humans. In particular, the substantial number of patients with overlapping neuropathologies will require a special attention as they might potentially benefit from therapies developed in the context of each

neuropathology. There is thus a great need to develop a better understanding of biomarkers that might inform on the biology of neuroinflammation in these patients.

Methods

Study design and data

Participants

Cases were selected from the Portuguese Brain Bank (PBB), obtained through appropriate consent procedures for the collection and use of human brain tissues for research. Criteria for inclusion for AD and LBD patients in the study were: (1) pure AD: 1) a clinical diagnosis of probable AD, 2) moderate or high levels of AD-pathology according to the revised guidelines of the NIA-AA association at autopsy[78] as well as AD-pathology equal or superior to Braak stage 5[79] and equal or superior to Thal stage 4[40] and 3) LB related pathology inferior to Braak stage 3[42]; (2) pure LBD: clinical diagnosis of probable DLB according to the DLB consortium criteria [18] or idiopathic PD according to the United Kingdom Brain Bank criteria[80] and Major Neurocognitive Disorder possibly or probably due to PD according to DSM-V criteria[81], and diffuse-neocortical or limbic-transitional LB related pathology at autopsy, with LB pathology equal or superior to Braak stage 4 and less severe levels of AD-pathology, equal or inferior to Braak stage 4[79]; (3) Mixed AD/LBD on neuropathological evaluation: 1) diffuse-neocortical or limbic-transitional LB related pathology was present at autopsy with LB pathology equal or superior to Braak stage 4, and 2) the levels of AD-pathology were intermediate or high according to the NIA-AA guidelines[78] as well as AD-pathology equal or superior to Braak stage 5[79] and equal or superior to Thal stage 4[40]. Exclusion criteria for both cases and controls were (1) heavy burden of other neurodegenerative pathologies associated with dementia such as frontotemporal lobar degeneration that meets the current pathological diagnostic criteria; (2) amygdala-predominant Lewy-related pathology; (3) presence of transactive response DNA binding protein 43 KDa (TDP-43) pathology beyond the entorhinal cortex (equal or above TDP-43 AD stage 3[82]); (4) presence of significant cerebrovascular disease and (5) incomplete medical information.

AD and control cases were selected in order to have similar age at death of the other two pathological groups less represented in the PBB collection (PD/PDD and LBD).

Clinical Information

Clinical information from cases relevant towards this study were collected from the medical records of the Portuguese Brain Bank. This included sex, age at onset of symptoms and age at death.

Immunohistochemistry

Neuroinflammation was characterized through immunohistochemistry in terms of microglial density using CD68 (a lysosomal glycoprotein associated with phagocytosis[5]) as a marker.

Tissue samples were focused on the middle frontal gyrus of the frontal cortex, a brain region frequently affected by ADNC and LBP.

The immunohistochemistry staining was performed using the Ventana OptiView DAB IHC detection kit and BenchMark Ultra processor (Ventana, Tucson, AZ, USA). Sections were cut at 6 μm thickness from formalin fixed, paraffin embedded blocks and mounted onto 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 (KP1, 1:300; Dako, Glostrup, Denmark) for 24 min at 36°C. Subsequently, the slides were incubated with OptiView universal HRP multimer (Ventana) for 8 min. 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).

Slide digitization

Slides were scanned using the 3DHistech P1000 digital pathology system to generate whole slide images (WSIs) at 20x. Scanning was performed by Ipatimup Diagnostics, that is accredited by the American College of Pathologists.[83] The WSI of each slide was further quality controlled in terms of image quality and labeling on the WSI viewer software 3DHistech CaseViewer. [83]

Selecting Regions of Interest

The WSI were imported on QuPath, a cross-platform open-source software for digital pathology and whole slide image analysis[84]. For each slide, the middle frontal gyrus was identified through its relative positioning to the superior frontal gyrus, that is systematically marked with an indentation by the Portuguese Brain Bank during tissue processing.

- Two representative regions of interest (ROI) within the middle frontal gyrus were selected from each slide corresponding to:
    - grey matter – entire area of grey matter in the middle frontal gyrus from the bottom of the sulcus to the tip of the middle gyrus on the side facing the superior frontal gyrus; a thin subpial area was discarded as meninges and its vessels were unevenly attached to the cortical surface.

- white matter – entire area of white matter within the gyrus.

Boundaries between white matter and grey matter were drawn and quality controlled by an experienced neuropathologist. A residual transitional area between white and grey matter with a variable thickness of around 100 to 200 µm that was not clearly imputable to either white or grey matter was discarded. A representative example of ROI selection using QuPath is shown in Figure 1 and 2.

Course debris with dimensions over 200 µm was discarded. Vessels with a size over 300 µm and their immediate surrounding area were discarded (vessels of this magnitude were only present in a few of the slides which could introduce bias).

The white and grey matter ROIs of each slide were exported to ImageJ with full resolution and saved as images in tag image file format (TIFF format). The considerable size of the ROI and high image resolution required ROI to be divided into smaller pictures, so as be able to process ROI with full resolution with the available computing power (representative picture in Figure 3).

Quantification of immunohistochemistry-stained area

Immunohistochemistry-stained area was quantified in terms of ratio of DAB stained area within ROI tissue area (in line with digital neuropathology methods reported in the literature[5,36]). A quantitative semi-automated method using ImageJ software was performed.

ROI tissue area calculation: the ROI RGB picture was converted into grey scale (8-bit conversion; Figure 4). The resulting picture had pixels with an intensity value from 0 (corresponding to absolute black) to 255 (corresponding to absolute white). In our dataset of pictures, the background was white, with most background pixels having a 255 intensity. Some pixels corresponding to background, for instance the inside of some small vessels, assumed values close to 255, but so did some pixels corresponding to tissue area. In this work, in order to include all tissue area and remove a maximum of background area, it was considered for all images that all pixels with an intensity of 254 or below corresponded to tissue area. Picture pixels with an intensity that was not absolute white (intensity values between 0 and 254) were therefore selected through image thresholding and assumed to correspond to tissue area. Tissue area in square micrometers was then calculated through the particle analysis ImageJ plugin. An image representation of the selected area was compared against the original RGB picture for quality control. In case of gross disparities, excess non-tissue area was manually eliminated, and tissue area recalculated.

DAB stained area calculation: the original ROI RGB picture was color deconvoluted in order to obtain separate independent pictures corresponding to its components, namely haematoxylin staining and DAB staining (Figure 5). Pictures corresponding to DAB staining were visually compared relative to the original RGB picture. An appropriate pixel intensity threshold that selected pixels with stronger DAB staining and excluded noise was manually selected after visually comparing threshold results versus original pictures for different samples in our dataset (Figure 6). The best threshold results were obtained for a threshold of 170. A threshold selecting pixels in the DAB staining picture with intensity between 0 and 170 was applied for all analyzed images. DAB stained tissue area in square micrometers was then calculated through the particle analysis ImageJ plugin.

Ratio of DAB staining was calculated as the ratio between the calculated DAB stained area and the calculated ROI tissue area.

For all cases and both regions of interest a minimum area of $4mm^2$ was analyzed.

All experimental procedures were done blinded for patient condition.

Statistical analysis

Data analyzed in this study was collected from the available clinical data provided by the Portuguese Brain Bank. Patients meeting the inclusion criteria and without exclusion criteria were selected. A total of 18 patients were selected. The patient sample consisted in terms of neuropathology groups of: 6 AD patients, 4 AD/LBD patients, 5 LBD patients and 3 controls. Information was collected with respect to each patient regarding sex, age at symptom onset, age at death and diagnosis. A sample of middle frontal gyrus was analyzed per patient in terms of CD68 protein load as described and data collected with regards to selected area of gray matter, selected area of white matter, CD68 protein load in gray matter, CD68 protein load in white matter. Apart from sex and diagnosis, that are categorical variables, all other variables are continuous.

A p value < 0.05 and a confidence interval of 95% were considered significant.

Characteristics of the sample of individuals in each group were described. Categorical variables were described using absolute frequencies. The Shapiro-Wilk test was used to assess the normality of the distribution of continuous variables. Given that all analyzed continuous variables had a normal distribution, they were described using mean and standard deviation. Homogeneity of variance across conditions was assessed using the Levene test ($p > 0.05$). In order to compare across more than 2 conditions, one-way ANOVA test was used with post hoc Tukey to correct for multiple comparisons. In order to compare CD68 staining in the grey and white matter of each individual, a paired two-tailed t-test was used.

Survival analysis was carried out, with time from symptom onset until the occurrence of death as the dependent variable. Diagnosis was used as the independent variable. To assess whether the difference between different survival curves was statistically significant, the Log-rank test was used.

Levene test and survival analysis were performed using IBM Statistical Package for the Social Sciences (SPSS). Remaining statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc.).

Ethics review

This research project was approved by the teaching department's coordinator, the CHUP's Administration Council, the entity responsible for access to information and the hospital's ethics committee. This research was also approved by the Portuguese Brain Bank and peer reviewed for funding by the scientific council of a foundation (Fundação Professor Ernesto Morais), which approved and funded this project. Patient data and samples from the Portuguese Brain Bank were collected through an informed consent appropriate for the collection and use of data and human brain tissue for research.

Results

Socio-demographic and clinical characterization

The study included 18 individuals from the Portuguese Brain Bank, of which 1 was female and 17 were male (Table I).

The selected individuals were distributed per neuropathological group: AD (n=6; mean [SD] age at death, 69.8 [5.9] years), AD/LBD (n=4; mean [SD] age at death, 64.0 [6.5] years), LBD (n=5; mean [SD] age at death, 72.8 [7.6] years), Control (n=3; mean [SD] age at death, 60.3 [12.0] years). The group of LBD patients included 3 DLB and 2 PDD. The only female individual in this study was included in the AD/LBD group. Clinical diagnosis in the AD/DLB neuropathological group included 3 patients with probable clinical AD and 1 patient with probable clinical DLB.

The average age of the first symptoms (either cognitive or neurologic of AD, PD or LBD) across groups was of 60.7±7.5 years (ranging from 51 to 77 years) and the average age at death was of 67.8±8.4 years (ranging from 48 to 81 years). The survival from symptom onset was on average of 8.5±5.6 years (ranging from 3 to 22 years). There were no statistically significant differences for these 3 variables between the neuropathological groups (respectively: one-way ANOVA p=0.2499; one-way ANOVA p=0.1426; Log Rank p=0.623).

Characterization of middle frontal gyrus CD68 protein load by immunohistochemistry

Selected gray matter and white matter regions of interest had on average an area of 22.9±11.3mm^2 and 13.2±6.9mm^2 (Table II) and did not have statistically significant differences between groups (ANOVA, p=0.8765 and p=0.7577 respectively). Of note, there seems to exist a small tendency towards lower selected area of gray matter in AD and AD/LBD relative to LBD and control. In terms of white matter, there also seems to exist a small similar tendency towards a lower selected area in AD and particularly AD/LBD relative to LBD. Interestingly in controls there was tendency for older age at death to correspond to a smaller selected area, both in the gray and white matter.

Gray matter

Middle frontal gyrus gray matter CD68 protein load assessed through the ratio of tissue area positive for CD68 in immunohistochemistry staining of *post mortem* human samples showed statistically significant difference between the studied groups of individuals (one-way ANOVA, p=0.0001; representative pictures of the 4 neuropathology groups in Figure 7 and 8; staining

quantification results represented in Figure 9). Statistically significant differences were found in pairwise comparison using Tukey post hoc test between the AD and control groups (average CD68 staining ratio of 2.2±0.6% for AD and of 0.8±0.3% for controls; p=0.0052) and between the AD and LBD groups (average CD68 staining ratio of 1.0±0.4% for LBD; p=0.0063). Statistically significant differences were also found between the AD/DLB and control groups (average CD68 staining ratio of 2.8±0.5% for AD/LBD; p=0.0006) and between the AD/DLB and the LBD groups (p=0.0006). No statistically significant differences were found between AD and AD/LBD or between LBD and control (respectively: p=0.3254 and p=0.9130). Although the quantified average differences in total stained area are relatively small between groups, these are validated visually in the immunohistochemistry microscopy images (Figure 7 and 8), in which pure AD and AD/LBD seem to have a considerably higher number of CD68 stained cells and stronger staining relative to pure LBD and control.

White matter

Middle frontal gyrus white matter CD68 protein load assessed through the ratio of tissue area positive for CD68 in immunohistochemistry staining showed no statistically significant difference between groups (one-way ANOVA, p=0.2675; representative pictures of the 4 neuropathology groups in Figure 10 and 11; staining quantification results represented in Figure 12).

Overall, white matter had a higher CD68 staining ratio than gray matter (average for all samples of 7.0±3.8% with a range of values between 1.8% and 15.6%; while for gray matter average for all samples was of 1.8±0.9% with a range of values between 0.4% and 3.3%). This difference is statistically significant (paired t-test: p<0.0001).

There seems to exist a small tendency towards higher CD68 protein load in AD and AD/LBD (average CD68 staining ratio of 9.0±4.6% for AD and of 7.8±4.3% for AD/DLB) relative to DLB and control (5.7±2.4% and 4.0±2.7% respectively). The highest CD68 protein load values reaching above 10% of tissue area staining were only found in AD and AD/LBD patients.

As suggested by staining quantification results, in the immunohistochemistry microscopy images, CD68 staining seems to be more intense in white matter regions, relative to gray matter regions.

Discussion

This exploratory study aims to better characterize neuroinflammation in *post mortem* brain samples of patients with Alzheimer's disease, Lewy Body Disease (Dementia with Lewy Bodies and Parkinson's Disease Dementia) and cases of co-pathology, in comparison with non-demented controls. In doing so we strive to generate new insight in human neuroinflammation and microglial activation, one of the most implicated biological mechanisms across common neurodegenerative disorders by transcriptomic and genome wide association studies[33,34,52]. This study is particularly relevant to the more than 60% of AD and DLB patients with mixed pathology[10] in which neuroinflammation mechanisms are considered to be largely unexplored.[12]

Mounting evidence points to an important and complex interplay of ADNC, LBP and neuroinflammation in patients with AD and LBD[12,15,36,54]. LBD is implicated in the literature to be influenced by accumulation of ADNC[85] and simultaneously by GBA associated accumulation of glucosylceramide[57]. Both ADNC and glucosylceramide are associated with an earlier onset and worse cognitive scores and risk of dementia in LBD[10,29,58]. Both ADNC and glucosylceramide are as well associated with increased microglial activation in LBD patients through *in vivo* PET imaging[12,58,63,64]. However, the mechanism through which they contribute to earlier symptom onset and dementia could be different in LBD patients with AD co-pathology and patients with pure LBD[54]. Recent GWAS data indicates DLB with ADNC have a different genetic risk architecture, with some important genetic risk factors for DLB, namely APOE and GBA, being reported to be specifically associated with mixed DLB and pure DLB respectively[54]. Of note, APOE ε4 frequency is also reported to be more associated with AD patients with LBP than in patients with pure AD[30]. Therefore, APOE is implicated in AD/LBD co-pathology relative to pure AD or pure LBD in patients with either a primary AD or a primary LBD neuropathology diagnosis. In contrast, GBA mutations are reported to be more frequent in pure DLB than in AD/LBP and to not be significantly more frequent relative to non-demented controls in AD[59]. These findings together suggest that AD, AD/LBD and LBD while all being associated with neuroinflammation, in particular microglia activation, might have different neuroinflammation dynamics and biology, which could have implications for future neuroinflammation targeted therapies for these neurodegenerative conditions, in particular for patients with mixed pathology.

In order to characterize microglial activation in co-pathology patients our study focused on CD68 protein load as this is a well-established microglial marker that is associated with microglial activation in humans[5,6,8,36,48]. CD68 protein load has been studied in both AD and LBD brains but to the best of our knowledge has not yet been specifically studied in AD/LBD human neuropathology[12].

Post mortem studies of patients with dementia, while providing direct evidence from human tissues and biology, are most representative of late stage pathology[12]. The present study focuses on the middle frontal gyrus that is implicated in neuroinflammation in AD and LBD but relatively spared from neurodegeneration compared to the other brain regions such as the temporal lobe[12,45,86] and could thus be a vantage point to study slightly less advanced neuroinflammation dynamics.

This work was based on tissue samples and clinical data from the Portuguese Brain Bank. Due to its exploratory nature and scarcity of clinically well characterized human brain samples, it involved a reduced number of samples leading to some limitations. Of note while these neurodegenerative conditions affect both males and females, there was only one female individual in the final group of 18 individuals included in the study. The patients in our study had as well overall an age of symptom onset that was younger than that of some cohorts of patients in other studies [5,10] and does not represent the epidemiology in the literature[87]. This could translate possible referral bias from subspecialized neurology consultation to the brain bank or possible differences in individuals who are more likely to voluntarily chose to enroll with informed consent in a brain bank.

In terms of survival from symptom onset, in a study by Chatterjee *et al* the group of patients with pure severe ADNC tends to have a longer survival relative to the group with pure severe LBD [10]. The same is verified in our study but only if PDD cases are excluded from the pure LBD group, as PDD patients were found to have a longer time of survival from the age of symptom onset relative to AD, while DLB have a tendency towards having a shorter time of survival relative to AD. Chatterjee *et al* does not specifically report the proportion of PDD and DLB patients in their cohort. This study was not designed with a focus on comparing between pure DLB and pure PDD patients. By definition, PDD have an onset of dementia that happens more than one year after symptom onset, while DLB have an onset of dementia within the first year of symptoms[3]. A longer disease duration for PDD could be associated to the expected longer span of time before onset of dementia, to a faster disease progression after dementia onset or to association with other co-morbidities or confounding factors that would need to be controlled for in a study specifically designed to explore differences between patients with pure PDD and pure DLB.

In both studies the group with combined severe AD/LBD co-pathology has a survival from symptom onset that is very close to that of the pure severe ADNC. Higher ADNC load in DLB is reported to correlate with shorter time from symptom onset to dementia onset, as well as to shorter survival time and a worse cognitive phenotype relative to lower ADNC load in DLB[29,85]. Though we did not find shorter survival in AD/LBD relative to pure LBD in our study, the very low number of cases, particularly of the AD/LBD group (which is also the only group to include a female individual), and

the fact that our LBD group was composed of both DLB and PDD may contribute to limitations in interpreting results.

In our study, all participants with AD/LBD had severe LBP, so it was not possible to validate the reported shorter survival associated with more severe LBP in AD/LBD patients described in the literature[10].

In the present work we established a semi-automated pipeline for whole slide image analysis of CD68 immunohistochemistry staining in white and gray matter of the middle frontal cortex of human *post mortem* samples, which involved several iterations of optimization and validation steps. This whole slide image approach enables the analysis of a whole region of interest rather than sampling of smaller representative pictures within regions of interest, contributing to more representative and reproducible results with lower user bias. The development of a semi-automated pipeline for staining quantification enables a more timely analysis of big high resolution whole slide microscopy images which would be otherwise difficult to analyze manually, also reducing user bias and increasing reproducibility [6,83]. Other strengths of our approach are the blinding to neuropathological group status of the person selecting areas of interest and the step of quality control of segmentation by an experienced senior clinical neuropathologist specialized in neurodegenerative disorders, also blinded to neuropathological group status.

Our approach to select full thickness grey matter samples from the sulcus to the apex of the gyrus and to select the whole white matter present in the analyzed section of the gyrus enabled us to obtain representative images that did not significantly differ in sampled area between groups. The tendency of middle frontal gyrus grey matter to have a slightly lower selected area in AD and AD/LBD patients might indicate a possible cortical atrophy in these patients relative to other groups. A larger sample or other type of more direct measurements would be required to infer and interpret such differences due to several possible confounding factors such as the exact angle of the tissue section and in some cases existence of parts of the slide that cannot be analyzed given the presence of artefacts. Another important confounding factor that would need to be controlled for in order to do such types of comparisons would be the age at death, considering that it is well documented in the literature that cortical atrophy occurs and correlates with age in older adults, namely from MRI studies[86]. Further analysis of brain weight at autopsy in our cohort data would also help to corroborate findings of brain atrophy.

A reduced cortical thickness in AD patient samples would be consistent with data from the literature reporting generalized cortical atrophy in AD, that also affect frontal brain regions such as the middle frontal gyrus [15,88]. Patients with severe ADNC (either pure or in co-pathology) were also reported in

the mentioned Chatterjee study to correlate with reduced brain weight even after controlling for age at time of dementia diagnosis and survival[10]. Chatterjee *et al* also report that more ADNC load is associated with more atrophy in AD/LBD patients[10]. According to the Chatterjee study a longer survival from diagnosis of dementia was associated with lower brain weight[10].

DLB are reported in the literature to have less pronounced global cortical atrophy relative to AD in structural brain imaging, even though atrophy can also be present, namely in frontal regions[12,88]. PD and especially PDD are reported to have cortical thinning in structural MRI that can affect the frontal regions[88]. The selected areas in LBD as a group, tended to be higher than those of AD, in accordance with an expected lower atrophy relative to AD from the literature.

Middle frontal gyrus gray matter of AD patients was shown to have statistically significantly higher CD68 protein load assessed through ratio of CD68 positive staining relative to both control individuals and to pure LBD patients. Pure LBD patients were not significantly different from the controls for this feature.

Higher CD68 protein load in AD brain samples is consistent with the literature in terms of *post mortem* studies[4,6,8] and the role microglia and neuroinflammation in AD pathology that genetic studies suggest[32]. Higher CD68 protein load in neuropathologically diagnosed AD patients relative to age matched non-demented controls in the middle frontal gyrus gray matter has already been reported in the literature using a pipeline that only analyzed representative sample pictures from this region of interest[36]. The semi-automated CD68 protein load pipeline established in the present work was able reproduce these results using a more representative whole slide image approach that analyses the whole region of interest, reducing possible sources of bias. This result validates the ability of our pipeline to detect differences in CD68 protein load previously reported in the literature in large cohorts, even in a preliminary exploratory study with a very reduced number of studied individuals.

Microglial activation in severe pure AD relative to controls has already been described in the literature in several brain areas, namely in the superior frontal cortex[89].

Absence of prominent difference in CD68 protein load between the pure LBD and control groups is also consistent with findings in the literature.

A recent *post mortem* study in DLB patients with low AD pathology found no difference in the temporal lobe in terms of CD68, Iba1 or HLA-DR protein load relative to control, which suggests absence of microglial activation in late stage DLB pathology[5]. Another *post mortem* study looking at the hippocampus of DLB patients with low ADNC also found no difference in Iba1 and CD68

relative to controls[6]. Several cortical areas (superior frontal cortex, inferior temporal cortex, anterior cingulate cortex, and entorhinal cortex), as well as the CA2-3 region of the hippocampus were also found to not differ in terms of HLA-DR protein load in pure DLB relative to controls, which also suggests absence of microglial activation[89]. These studies seem to indicate a generalized absence of prominent microglial activation in pure DLB at *post mortem*, which is consistent with the *in vivo* TSPO PET data in DLB patients that points to a waning microglial activity in late stage disease[12,64].

There are *post mortem* studies in the literature that report higher CD68 protein in the substantia nigra and hippocampus relative to controls in cohorts of iLBD and PD/PDD patients with only mild ADNC[70,71]. There are also some TSPO PET studies in PD/PDD patients that suggest microglial activation across several cortical areas from an early stage in PD that persists in time in PDD[65,66]. However, in TSPO PET studies, only the midbrain is consistently implicated in studies with first and second-generation ligands[66]. There are also reports of persistence of some microglial markers in PD/PDD that, unlike CD68 protein load, do not seem to correlate with clinical features at the *post mortem* stage[70]. Different microglial markers are also reported to not always correlate, representing diversity within microglial states, with implications for clinical phenotype correlation[36,50]. It is thus plausible for an absence of CD68 protein load to be found at the *post mortem* late stage in frontal brain regions such as the middle frontal gyrus, that are not as implicated in PD/PDD pathology as the midbrain and temporal lobe[42,66,88].

The absence of prominent CD68 protein load in pure LBD relative to controls in the middle frontal gyrus in post mortem late stage pathology context found in the present study, suggests that if there is neuroinflammation in this context, it is likely that microglial activation and phagocytic activity may have a limited role in it[48].

Other neuroinflammation and pathological processes such as senescence and dystrophic microglia, astrogliosis and T-lymphocyte recruitment may however be present, as is implicated in the literature for the midbrain and temporal lobe[12,67].

The most important finding in this study was that in human middle frontal gyrus gray matter, which was shown to differ in terms of neuroinflammation between pure AD and LBD patients, the patients with AD/LBD co-pathology had a neuroinflammation pattern that resembled the pure AD group and differed significantly from the LBD group in terms of CD68 protein load. This finding was consistent among the 3 co-pathology patients with probable AD phenotype and primary AD neuropathological diagnosis, as well as for the co-pathology patient with probable DLB clinical phenotype and primary DLB neuropathological diagnosis.

This new preliminary insight is in line with the mounting evidence that while the pathologies of pure AD, AD/LBD and pure LBD are all implicated in neuroinflammation[12,44,45,63,64], they might differ in particular neuroinflammation dynamics in accordance with their reported difference in genetic architecture[30,33,52,54,59]. It also suggests a similar neuroinflammation phenotype for co-pathology patients with primary AD or primary LBD clinical phenotype, in accordance to evidence of shared genetic architecture from GWAS[54] and genetic studies[30,59]. The scope of this preliminary study is limited to one neuroinflammation marker, in one brain region, in a reduced number of late-stage patients. It is nevertheless an early proof of concept of distinct neuroinflammation patterns in patients with AD/LBD co-pathology that could be very relevant and informative towards future clinical trials targeting neuroinflammation[74] in more than 60% of AD and LBD patients[10].

In our study, CD68 staining in the middle frontal gyrus gray matter, despite being higher in terms of mean value in AD/LBD relative to pure AD, was not statistically different between the AD/LBD and the pure AD neuropathology groups. This data could suggest a prevailing effect of ADNC in this context, as there was not a statistically significant difference between pure AD and AD/LBD. This data could potentially still be compatible with a possible additive effect of ADNC and LBP pathology in terms of their association with microglial activation, as in vivo TSPO PET studies indicate a waning of microglial activity in late stage DLB disease.[12,64] In *post mortem* late stage pathology, DLB is reported to not have substantial microglial activation in the temporal lobe but rather increased dystrophic microglia[5], which may also be the case in the medial frontal gyrus. Our current study's sample size may also not be big enough to detect a small tendency towards higher CD68 in AD/LBD associated with a residual or lingering effect of LBP in microglial activation. Another possibility is that in cases with very high ADNC and correspondingly high CD68 and microglial activity, as in the present study, LBP may no longer be able to contribute to an even higher microglial activity. In order to address this issue, it would be important to confer if LBP load has an effect in terms of CD68 and/or other microglial activation markers in AD cases with moderate or lower ADNC load, relative to pure AD cases with equivalent levels of ADNC.

Higher CD68 protein load, is associated with higher microglial phagocytic activity[12,37,48] and in the context of late stage AD is proposed to be associated with a deleterious pro-inflammatory phenotype and worse clinical profile[36,41,50]. Higher CD68 protein load in the middle frontal cortex in particular, is also reported in the literature to correlate with AD clinical dementia status, poor cognitive function and ADNC pathology[36,47]. Higher or non-inferior CD68 protein load would thus be plausible in AD/LBD neuropathology, given that AD/LBD are reported to have a worse phenotype relative to pure AD[10]. The literature also reports worse phenotype in a cohort of AD patients for those who had LBP[10,30], which further supports the plausibility of higher CD68 protein load in AD

patients that have AD/LBD neuropathology. Considering that, at least with the current number of cases studied, it was not possible to detect a significant difference in CD68 protein load in AD patients with AD/LBD co-pathology relative to pure AD in the middle frontal gyrus, there could be other neuroinflammatory pathological mechanisms involved or areas affected that were not accounted for in this very preliminary and exploratory study, in particular T cell recruitment and microglial senescence and dystrophic microglia that are implicated in LBD *post mortem* studies[12].

The AD/LBD patient with probable clinical DLB did not seem to stand out from the other 3 patients with probable clinical AD in the AD/LBD group in terms of age at symptom onset, age at death or disease duration. It also did not seem to differ in terms of *post mortem* CD68 staining ratio in the middle frontal gyrus. It is not possible to compare AD/LBD patients with primary AD or primary LBD clinical and neuropathological phenotype in this study due to the small sample size. A similar biology of AD/LBD and LBD/AD would be aligned with the data from GWAS and genetic studies that point to a similar genetic architecture in cases of mixed AD/LBD pathology, namely in terms of higher contribution of APOE4 to the pathology of this group of patients[30,54,59]. *In vivo* amyloid beta PET data of worse cognitive phenotype in DLB patients with higher amyloid load also support a plausible stronger neuroinflammatory phenotype in these patients[29]. According to a very recent review on neuroinflammation in DLB patients only one *post mortem* study in the literature has so far found increased CD68 protein load in the DLB brain (in the temporal lobe and superior frontal gyrus; that could arguably be associated with microglial senescence rather than microglial phagocytic activity based on morphological findings according to the authors of the study)[12,49]. Of note, unlike other reported *post mortem* studies in the DLB literature, the study that reported to find an increase in CD68 protein load in DLB did not control for ADNC in terms of amyloid beta burden[12,49]. A similar contrast is described in the literature regarding two *post mortem* studies that did not control for ADNC and found an increase in the microglial marker HLA-DR in several cortical regions of DLB brain relative to control, that were not replicated in a different post mortem study in DLB that excluded cases with significant ADNC[5,89]. The literature could thus be corroborating our finding of high CD68 protein load in our DLB/AD case, but not in the pure LBD group, suggesting an association of microglial activation or senescence with ADNC load in DLB patients with AD co-pathology.

Middle frontal gyrus white matter was not found to have statistically significant differences in CD68 protein load between AD, AD/LBD and LBD patients.

This finding is consistent with the literature, as white matter has been reported in a systematic review of AD post mortem studies to be particularly resistant to AD associated inflammation[47],

including in the particular context of CD68 protein load in frontal brain regions (increased CD68 protein load in AD relative to control found in frontal brain region white matter only in 1 of 3 post mortem studies considered in this systematic review[47]). To the best of our knowledge, no differences in CD68 protein load in middle frontal gyrus white matter have been reported in the LBD human *post mortem* study literature[12,67,68].

In the present study, the patients with the highest levels of CD68 protein load in the middle frontal gyrus white matter were found only in the AD and AD/LBD groups, which could suggest a higher CD68 protein load in both of these groups in line with findings for middle frontal gyrus gray matter. If this tendency were to be confirmed in a larger study, a similar trend in the microglial findings of white and gray matter would strengthen the case for the existence of distinct neuroinflammation patterns in patients with AD/LBD co-pathology.

White matter was found relative to gray matter to have a higher CD68 protein load in all individuals and study groups, as expected and reported in the literature[8,47,49], which further supports the ability of our digital neuropathology quantification method to reproducibly detect differences in staining.

Limitations, strengths and future directions

The present study, in part due to its preliminary and exploratory nature, has some limitations that should be considered. As mentioned, our study has a small sample size, underrepresentation of females and a selection bias due to recruitment from a brain bank with a possible bias towards patients with more severe, earlier onset pathology. The retrospective nature of the study, in which clinical data is based on medical records from the brain bank, may limit the quality of our assessment of the age of symptom onset, as early symptoms may have been underreported. This could be particularly relevant in patients with memory impairment, as they may no longer be a reliable source of clinical information at time of referral and some information may need to be obtained through third persons (eg. spouses, family members or caretakers), introducing further bias. In future studies, a prospective study approach would reduce some of these limitations.

Neuropathology staging of ADNC and LBP, used to select patients during recruitment, relies on scales that do not have 100% inter-rater agreement[18,78,90], and was performed by different neuropathologists from the brain bank which could also introduce some minor variability in neuropathological staging of patients. A possible way to reduce this bias would be to stage LBP with staging systems such as Lewy Pathology Consensus criteria (LPC)[90], that have been shown in the literature to have higher inter-rater agreement rates, being more robustly reproducible[90]. For even

finer comparison, digital pathology could be used to detect small differences across vast brain regions with reduced user bias, as reported in a recent *post mortem* study in the literature[68] in an approach similar to the one used in the present study for CD68 protein load quantification. In small cohorts, another more exhaustive alternative approach reported in the literature is the complete enumeration and sorting for unlimited sectors (CENSUS) approach[91].

In this study, LBD patients, both DLB and PDD, were included in the same study group as they both are characterized by LBP and their differentiation is not consensual in the literature due to absence of definite pathological criteria to discriminate them[3]. This could contribute to heterogeneity within the group, particularly in terms of clinical features but possibly also in terms of neuropathology, as some differences between DLB and PDD have been reported[25,27]. In future studies with a greater sample size of patients, it would be important to also study DLB and PDD independently in terms of the influence of ADNC and LBP co-pathology in neuroinflammation.

A strength of our study is the semi-quantitative analysis of CD68 immunohistochemistry images using whole slide images that reduces user bias and increases representativity of the area analyzed within the regions of interest[68,83,84]. Blinding of the person selecting the regions of interest to the study group and quality control of segmentation of regions of interest by an experienced neuropathologist also blinded to the study group also contributes to reduce bias and increase reproducibility of results. In this semiautomated whole slide approach it was not possible to completely exclude vessel area, so there might be a residual contribution of perivascular macrophages, that would be expected to stain for CD68[47]. This contribution is expected to be equivalent in cases and controls and thus controlled for when interpreting results. Peripheral macrophages are reported to mostly not enter the central nervous system in the context of neurodegenerative disorders such as Alzheimer's Disease, and are thus also not expected to directly influence results[92]. A thin subpial area in gray matter area and a thin transitional area between gray and white matter were not analyzed in this study. It would be important in future studies to exclude prominent contribution of these regions to differences between groups. Clustering of neuropathological findings within regions of interest, for instance near ADNC and LBP, vessels or borders of the regions, might also be important in future studies for finer interpretation of results. Neuroinflammation findings such as microglial density and activation, astrogliosis and T-cell infiltration may potentially associate with particular subregions within ROI in some of the study groups[8,48,67].

CD68 load is suggested in the literature to not only reflect microglial activity but also to reflect aging dependent lysosomal accumulation of lipofuscin in senescent microglia[48,49]. In the particular

context of the middle frontal gyrus in AD patients, which has been extensively studied using different microglial markers and correlating with neuropathology and clinical features[47], namely by Minett *et al*[36], it is plausible that CD68 load could be reflecting at least in part an increase in microglial and phagocytic activity. It would be important to further characterize samples in terms of senescence markers such as p21 or lipofuscin for greater clarity on the interpretation of results, particularly as microglial senescence is also implicated in the literature to contribute to AD and LBD pathology[12,93] and could be differentially affecting pure AD and AD/LBD and LBD. As CD68 protein load is reported to increase with normal ageing[49], age at death could thus be a potential confounding factor when analyzing CD68 protein load. As there was no statistically significant difference in terms of age at death between study groups in our data, its contribution to bias in our study should be limited.

Microglia are reported in the literature to have dynamic and diverse phenotypes in response to their microenvironment and to have different transcriptional profile clusters in humans, namely in single cell RNA sequencing studies.[94] For a more comprehensive understanding of microglial contribution to differences in neuroinflammation between AD, AD/LBD and LBD it would be important to assess other microglial markers associated with the different microglial transcriptional clusters described in humans to be implicated in neurodegeneration[67,94]. It would in particular be important to characterize Iba1 expression as it has already been reported to not correlate with CD68 protein load in the middle frontal gyrus in terms of clinical neuropathological associations in a study of microglia in an cohort of AD patients[36]. Iba1 has also been implicated in some LBD post mortem studies[12,67,68]. In further studies it would additionally be important to confirm microglial activation findings by analysis of microglial morphology, since the proportion of morphologically active microglia (PAM) and different types of dystrophic microglia have been reported to correlate strongly with ADNC and cognitive decline[6,35], and could therefore be more sensitive in exposing the particularities of the neuroinflammation biology in each condition.

Exploring differences in terms of gene expression (for instance through RNA sequencing) might enable in future studies the correlation of the presence of subpopulations of microglia with clinical and neuropathological features[94]. Future work to further characterize the dynamics of microglia activation in patients with (simultaneous) presence of different neuropathologies may include single-cell or single-nuclei RNA profiling, which have shown promise as effective tools to obtain a deeper insight into the heterogeneity of microglia sub-populations[13,50,67,95]. In particular single-cell RNA sequencing seems to be the most promising approach in terms of obtaining a faithful RNA profile for some of the relevant genes in microglia cell activation[96]. There are currently lines of research investigating new avenues for therapies based on restoration of healthy microglial

transcriptional signatures recently reviewed and summarized by Matsudaira *et al*[94]. Knowing clinical correlates of the shift of microglia towards different specific pathological transcriptional profiles would enable a more accurate prediction of patients that would most benefit from each of these new therapeutic approaches currently in development. Current literature indicates that murine microglial subpopulations do not fully represent those found in human samples[94], further indicating the need for more insight into disease specific microglial signatures in humans.

Deeper phenotyping of the cohort would be very important to control for more confounding factors such as co-morbidities. It would also allow for better characterization of clinical differences between study groups and to correlate neuropathological findings with more relevant clinical phenotypes. Identifying correlations between neuroinflammation phenotypes in different patient groups and clinical outcomes could possibly be informative for future clinical trials targeting neuroinflammation.

Looking at other brain areas and neuroinflammation phenotypes such as T-lymphocyte infiltration and astrogliosis that are implicated in AD and LBD pathology[8,12,67] would also be very relevant in future studies for a more comprehensive understanding of the biology of neuroinflammation in these conditions.

It would be important to control for the most relevant genetic variants with higher frequency and effect size implicated in AD and LBD pathology and neuroinflammation, namely those present in APOE, BIN1, GBA, SCNA, and TMEM175[54]. This would be particularly important when studying patients with co-pathology, given the reported higher contribution of deleterious APOE variants in the genetic architecture of AD/LBD[30,54], that could contribute to worse clinical phenotype namely through influence in neuroinflammation dynamics[51]. A recently reported genetic variant (rs2997325T) that showed genome-wide association with the proportion of activated microglia in human neuropathology and TSPO PET findings[35] would also be worth consideration. In future studies with a greater sample size, cross analysis of these polymorphism with patient records, clinical phenotype and histological findings would possibly contribute to a finer interpretation of their influence in AD and LBD pathology.

GBA is reported to be especially implicated in pure DLB pathology[54] as well as in neuroinflammation in *in vivo* TSPO PET studies[58]. Glucosylceramide accumulation is additionally implicated to potentiate LBP accumulation[57]. Assessing glucosylceramide influence in ADNC, LBP and neuroinflammation might thus be informative in clarifying differences in neuroinflammation between LBD and AD/LBD in future studies.

Ultimately, integrating the insight of future studies into the particularities of the neuroinflammation biology of patients with ADNC and LBP co-pathology and their clinical correlations will help identify relevant biomarkers for a more personalized and effective therapeutic approach to these patients that account for a considerable share of patients with dementia.

Conclusions

An established neuroinflammation marker for microglial activation, CD68, was studied in human *post mortem* brain tissue in a preliminary experiment specifically designed to compare both AD and DLB patients with pure pathology and co-pathology. A small cohort of 18 neuropathologically characterized patients with AD, AD/LBD and LBD pathology were selected from the Portuguese Brain Bank and assessed.

A semi-automated whole slide image analysis digital neuropathology pipeline was established and optimized for the quantification of CD68 immunohistochemistry staining in white and gray matter of the middle frontal cortex of human *post mortem* samples. This approach was sensitive enough to replicate reported literature findings of higher CD68 protein load in human middle frontal gray matter of AD patients relative to controls even in an exploratory study with small sample size.

While no differences were found in CD68 protein load in pure LBD patients relative to controls for this brain region, the AD/LBD pathology group was found to have higher CD68 protein load relative to controls at levels equivalent to those of AD patients. In this later group a clinical DLB case was included. This preliminary data may help clarify differences between studies in the DLB literature that inconsistently suggest increased microglial activity in DLB in human *post mortem* studies.

Patients with AD/LBD pathology were found to resemble patients with pure AD pathology in terms of CD68 protein load in middle frontal gyrus gray matter. This data suggests a prevailing effect of ADNC in neuroinflammation pattern of patients with AD/LBD pathology, regardless of primary AD or LBD clinical features.

These data and the know-how for the neuropathological neuroinflammation study generated will enable further characterization of neuroinflammation dynamics in human *post mortem* brain samples of AD/LBD patients that account for more than 60% of AD and LBD patients and could be informative to promising neuroinflammation targeting novel therapies currently in clinical trials.

Disclosure Statement

There are no conflicts of interest to disclose.

Tables and Figures

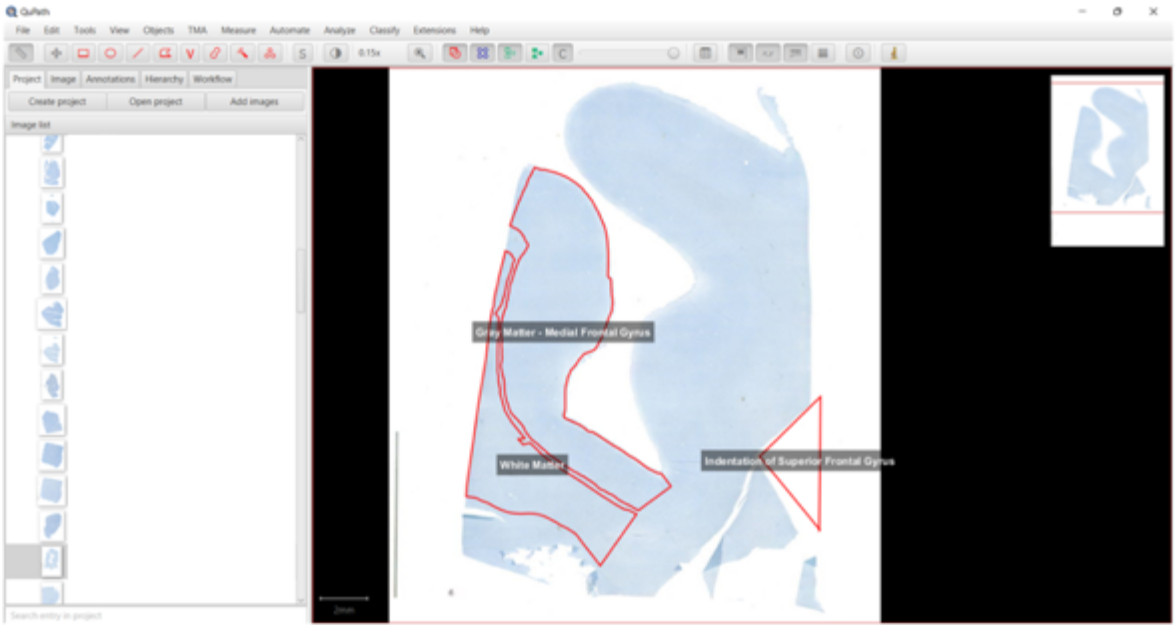


Figure 1 - Region of interest selection within the middle frontal cortex on a representative whole slide image on QuPath

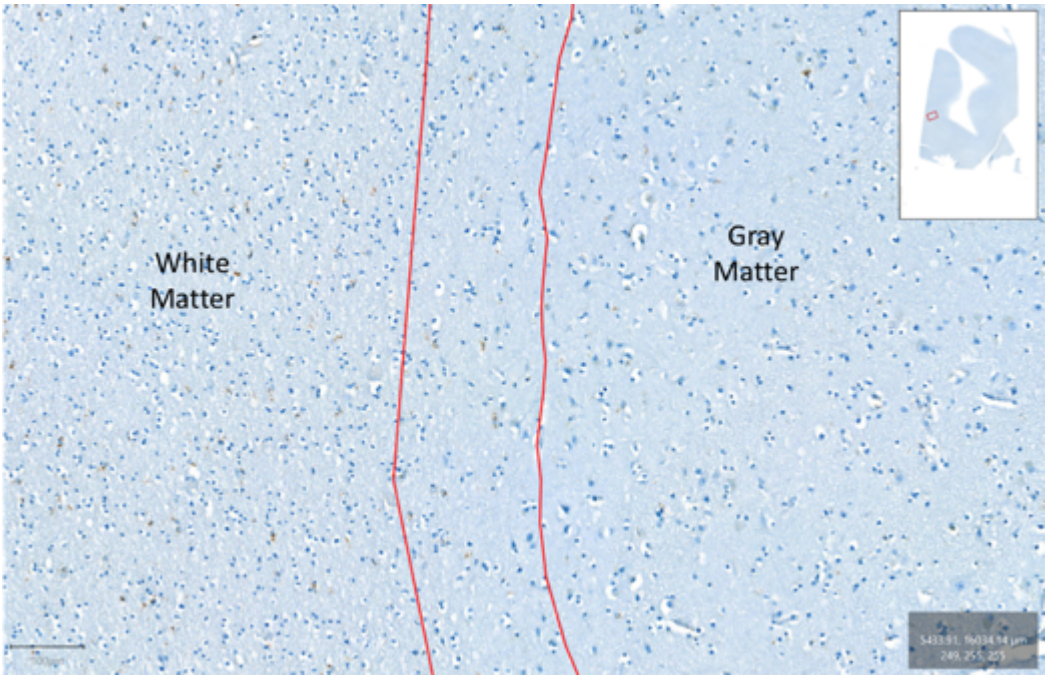


Figure 2 – Representative image of white and gray matter segmentation during ROI selection on QuPath

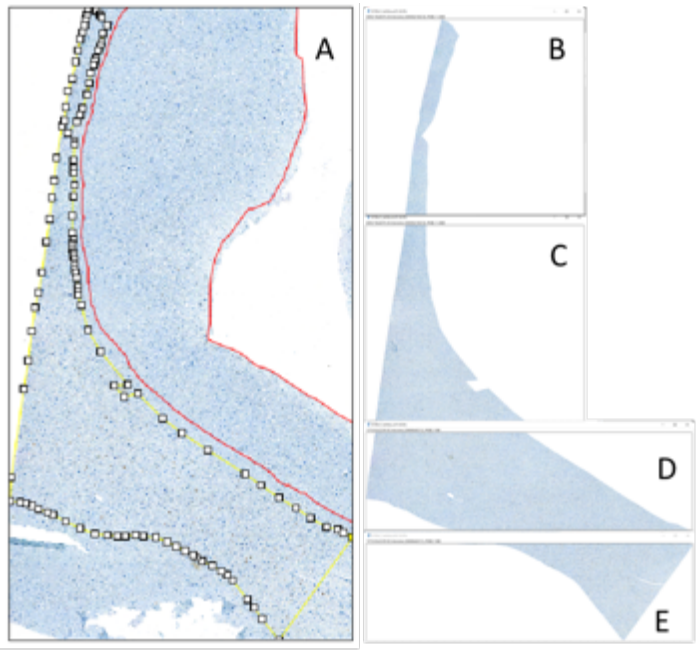


Figure *3* – *Example of a region of white matter within a whole slide image of middle frontal cortex exported from QuPath (A) and imported into ImageJ in smaller images (B to E; amenable to image processing with the available computing power).*

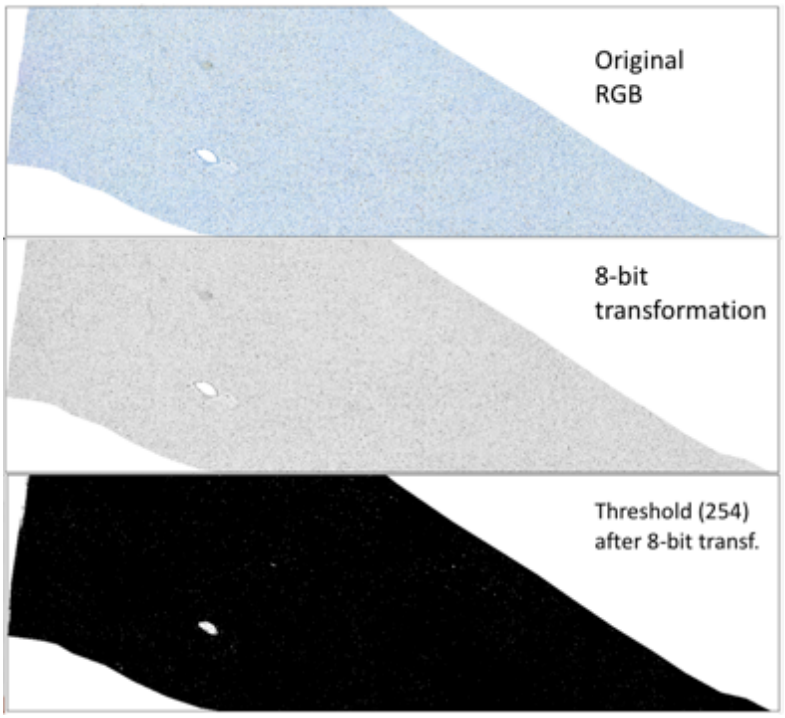


Figure 4 – *Semi-automated selection of tissue area through thresholding*

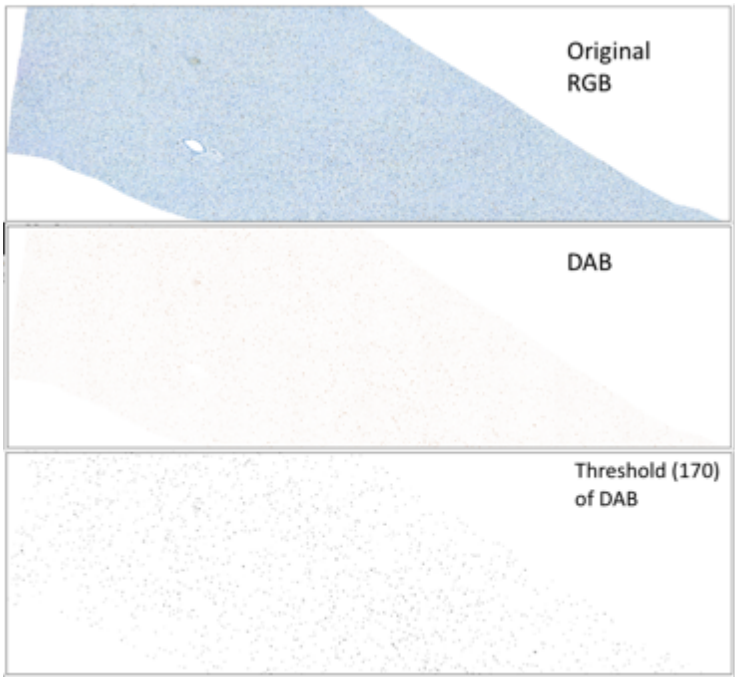


Figure 5 – Semi-automated selection of 3,3'-Diaminobenzidine (DAB) stained area through color deconvolution and thresholding

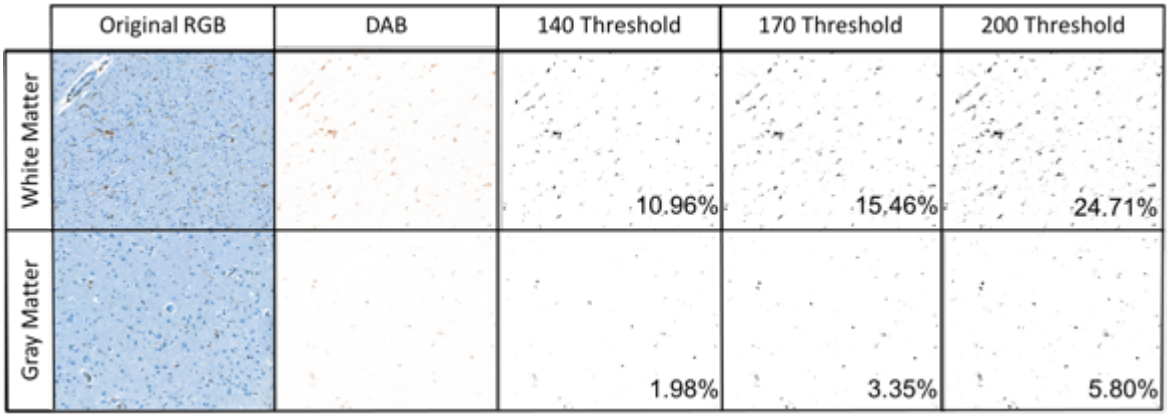


Figure 6 – Comparison of pictures of 3,3'-Diaminobenzidine (DAB) stained area selected using different intensity thresholds in representative white and gray matter images of a case with AD. Ratio of DAB stained area quantified by the particle analysis ImageJ plugin are plotted on the bottom right of each picture.

Table I – Demographic and clinical features of the individuals assessed

Neuropathological Group	AD	AD/LBP	LBD (DLB & PDD)	Control	p value
N	6	4	5	3	-
M:F	6:0	3:1	5:0	3:0	-
Age at Symptom Onset (years)*	62.7±7.5	55.3±3.1	62.8±9.0	NA	0.2499
Age at Death (years)*	69.8±5.9	64.0±6.5	72.8±7.6	60.3±12.0	0.1426
Survival from symptom onset (years)*	7.2±3.0	8.8±4.4	10.0±8.9	NA	0.623

*Values are expressed as mean ± SD. Age of Symptom onset corresponds to the age of onset of first symptoms either cognitive or neurologic of Alzheimer's Disease (AD), Parkinson's Disease (PD) or Dementia with Lewy Bodies (DLB). Average age at death for all groups was of 67,8±8.4 years. Groups were compared for these features. A p value was calculated by one-way ANOVA for age at symptom onset and age at death. A p value was calculated by Log Rank for survival from symptom onset. No statistically significant differences were found between the groups for these variables.

Table II – Area of selected gray and white matter in regions of interest

ROI	Neuropathological Group	AD	AD/LBP	LBP	Control	P value
White Matter	Area of ROI (mm^2)*	14.40±8.85	10.29±7.93	15.11±5.64	11.66±6.82	0.7577
	Min Value	4.59	4.04	7.62	5.10	-
	Max Value	28,87	20.78	22.75	18.71	-
Gray Matter	Area of ROI (mm^2)*	20.71±14,03	20.98±8.92	26.20±14.11	24.57±9.40	0.8765
	Min Value	9.32	8.77	13.52	14.90	-
	Max Value	46.81	29.47	48.47	33.67	-

*Values are expressed as mean ± SD. P value calculated by One-way ANOVA. Patient groups compared included: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease; n=6), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease; n=4), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease; n=5), control (individuals without clinical dementia at age of death; n=3).

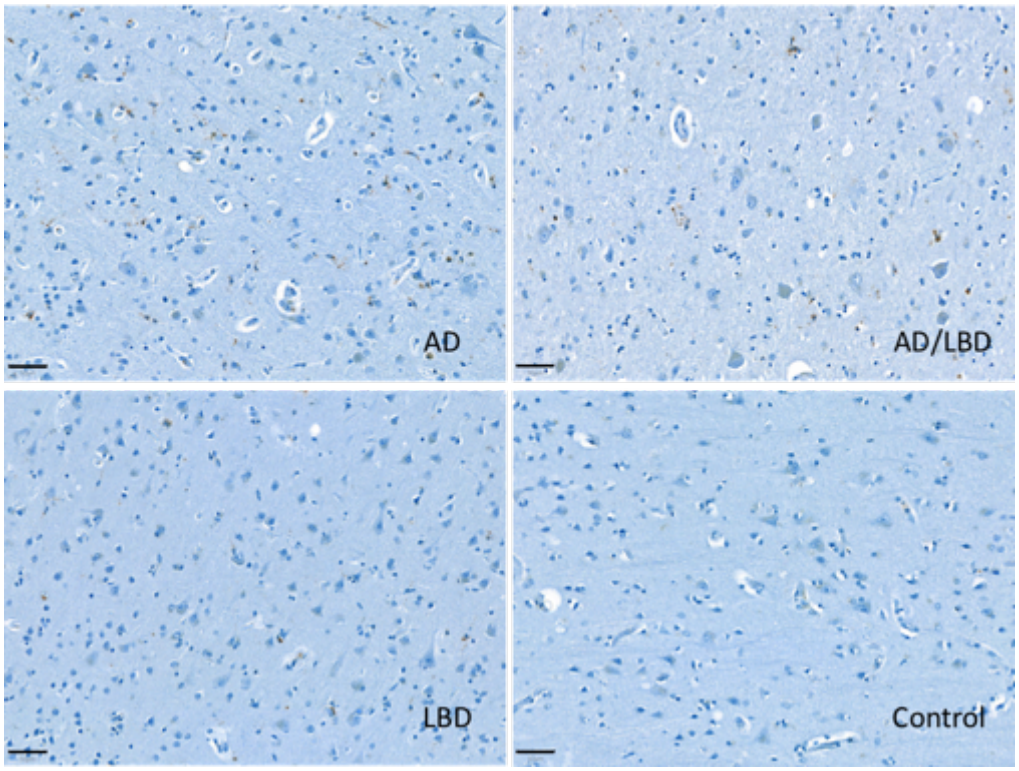


Figure 7 - Representative middle frontal gyrus gray matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 50 µm.

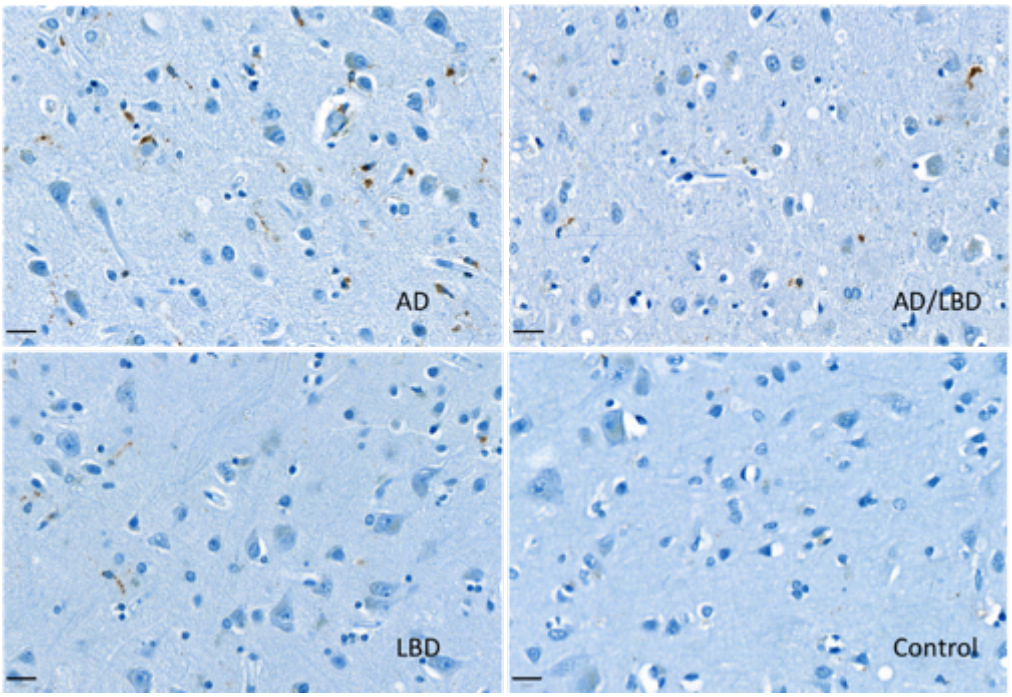


Figure 8 - Representative middle frontal gyrus gray matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 20 µm.

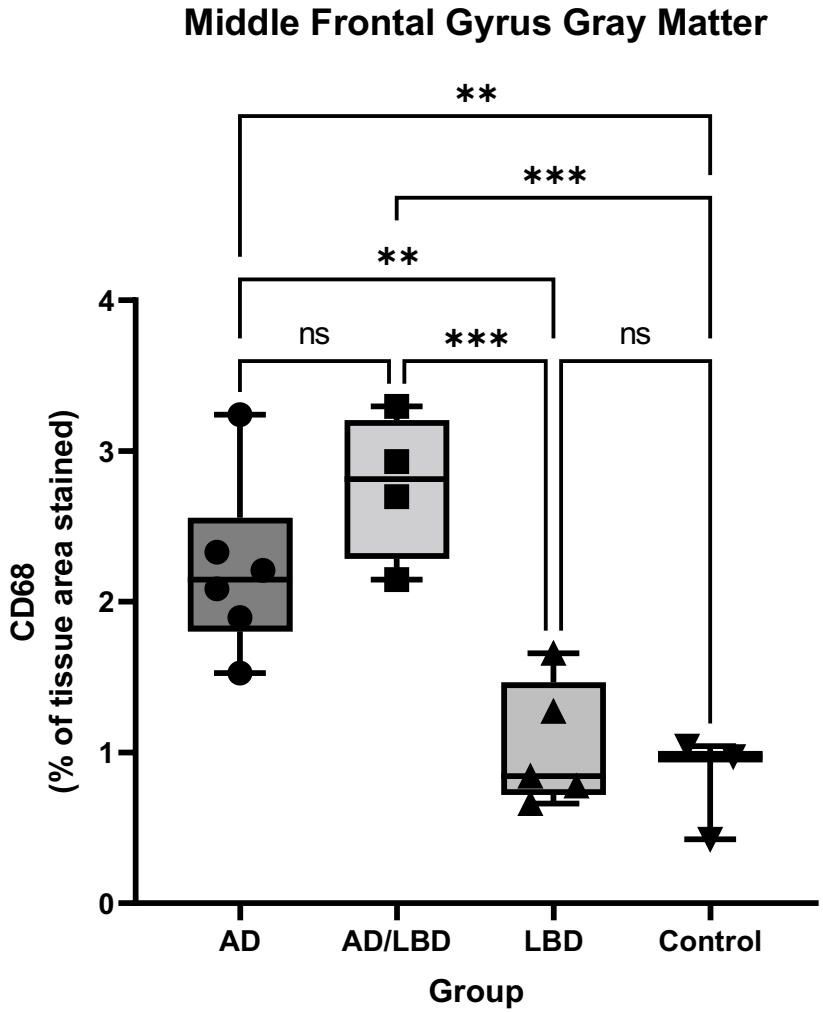


Figure 9 – Middle frontal gyrus gray matter CD68 protein load assessed through the ratio of tissue area positive for CD68 in immunohistochemistry staining of *post mortem* human samples. Patient groups compared included: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease; n=6), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease; n=4), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease; n=5), control (individuals without clinical dementia at age of death; n=3). Results of each of the groups for percentage of CD68 tissue staining are plotted in a boxplot representation. Asterisks indicate statistical significance (one asterisk corresponds to $P \leq 0.05$; two asterisks to $P \leq 0.01$; three asterisks to $P \leq 0.001$) and ns indicates no statistically significant differences were found ($P > 0.05$).

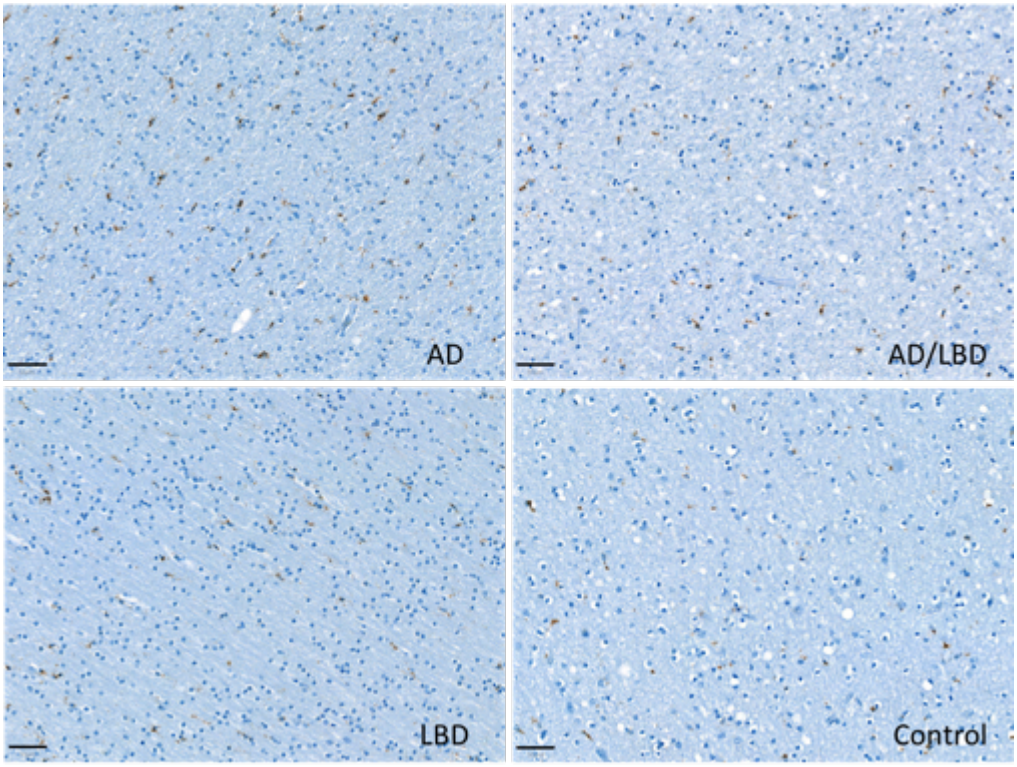


Figure 10 - Representative middle frontal gyrus white matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 50 µm.

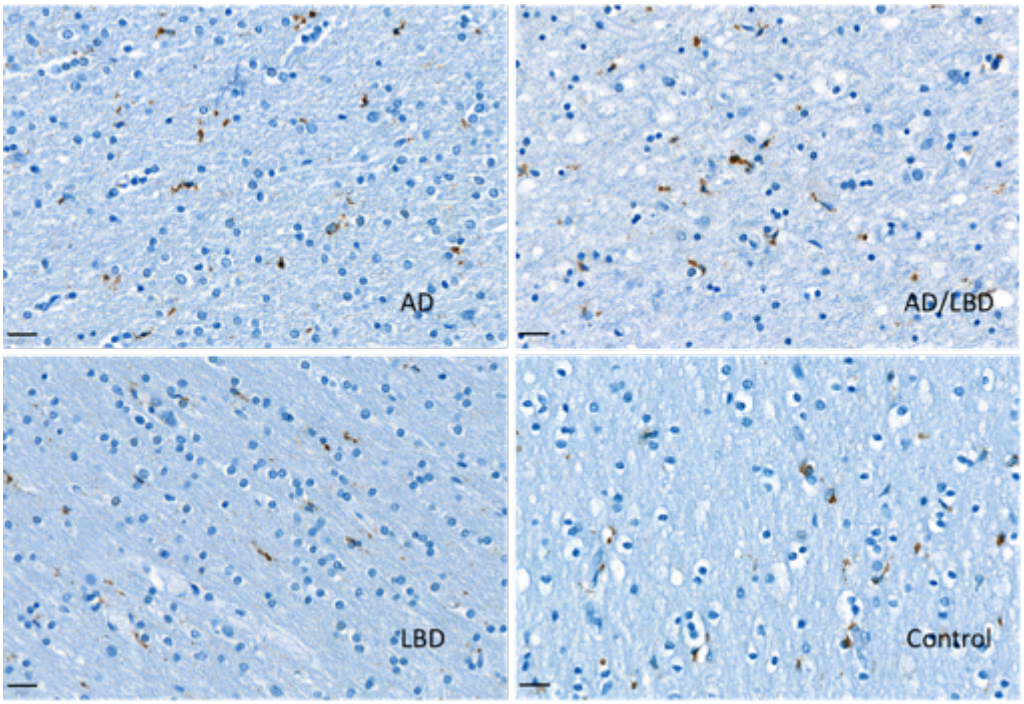


Figure 11 - Representative middle frontal gyrus white matter CD68 immunohistochemistry images of the 4 neuropathology groups: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease), control (individuals without clinical dementia at age of death). The scale bar represents 20 µm.

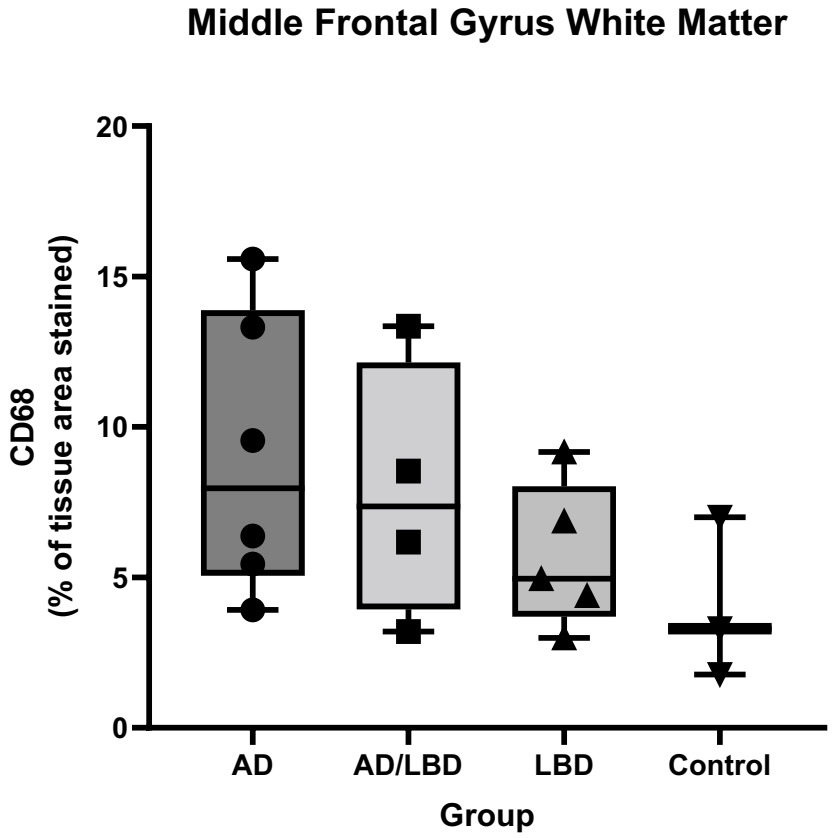


Figure 12 - Middle frontal gyrus white matter CD68 protein load assessed through the ratio of tissue area positive for CD68 in immunohistochemistry staining of *post mortem* human samples. Patient groups compared included: AD (patients with neuropathological criteria for Alzheimer's Disease but without neuropathological criteria for Lewy Body Disease; n=6), AD/LBD (patients with neuropathological criteria for both Alzheimer's Disease and Lewy Body Disease; n=4), LBD (patients with neuropathological criteria for Lewy Body Disease but without neuropathological criteria for Alzheimer's Disease; n=5), control (individuals without clinical dementia at age of death; n=3). Results of each of the groups for percentage of CD68 tissue staining are plotted in a boxplot representation. No statistically significant differences were found between groups (ANOVA, p=0.2675).

References

1. WHO. *Risk Reduction Of Cognitive Decline And Dementia*.; 2019. https://www.who.int/mental_health/neurology/dementia/risk_reduction_gdg_meeting/en/.

2. Nichols E, Szoeke CEI, Vollset SE, et al. Global, regional, and national burden of Alzheimer's disease and other dementias, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol*. 2019;18(1):88-106. doi:10.1016/S1474-4422(18)30403-4

3. Taipa R, Pinho J, Melo-Pires M. Clinico-pathological correlations of the most common neurodegenerative dementias. *Front Neurol*. 2012;MAY(May). doi:10.3389/fneur.2012.00068

4. Taipa R, Brochado P, Robinson A, et al. Patterns of Microglial Cell Activation in Alzheimer Disease and Frontotemporal Lobar Degeneration. *Neurodegener Dis*. 2017;17(4-5):145-154. doi:10.1159/000457127

5. Amin J, Holmes C, Dorey RB, et al. Neuroinflammation in dementia with Lewy bodies: a human post-mortem study. *Transl Psychiatry*. 2020;10(1). doi:10.1038/s41398-020-00954-8

6. Bachstetter AD, Van Eldik LJ, Schmitt FA, et al. Disease-related microglia heterogeneity in the hippocampus of Alzheimer's disease, dementia with Lewy bodies, and hippocampal sclerosis of aging. *Acta Neuropathol Commun*. 2015;3:32. doi:10.1186/s40478-015-0209-z

7. Heneka MT, Carson MJ, Khoury J El, et al. Neuroinflammation in Alzheimer's disease. *Lancet Neurol*. 2015;14(4):388-405. doi:10.1016/S1474-4422(15)70016-5

8. Taipa R, Ferreira V, Brochado P, et al. Inflammatory pathology markers (activated microglia and reactive astrocytes) in early and late onset Alzheimer disease: a post mortem study. *Neuropathol Appl Neurobiol*. 2018;44(3):298-313. doi:10.1111/nan.12445

9. Matej R, Tesar A, Rusina R. Alzheimer's disease and other neurodegenerative dementias in comorbidity: A clinical and neuropathological overview. *Clin Biochem*. 2019;73(August):26-31. doi:10.1016/j.clinbiochem.2019.08.005

10. Chatterjee A, Hirsch-Reinshagen V, Moussavi SA, Ducharme B, Mackenzie IR, Hsiung GR. Clinico-pathological comparison of patients with autopsy-confirmed Alzheimer's disease, dementia with Lewy bodies, and mixed pathology. *Alzheimer's Dement Diagnosis, Assess Dis Monit*. 2021;13(1):1-7. doi:10.1002/dad2.12189

11. Schneider JA, Arvanitakis Z, Bang W, Bennett DA. Mixed brain pathologies account for most dementia cases in community-dwelling older persons. *Neurology*. 2007;69(24):2197-2204. doi:10.1212/01.wnl.0000271090.28148.24

12. Amin J, Erskine D, Donaghy PC, et al. Inflammation in dementia with Lewy bodies. *Neurobiol Dis*. 2022;168(October 2021):105698. doi:10.1016/j.nbd.2022.105698

13. Lewcock JW, Schlepckow K, Di Paolo G, Tahirovic S, Monroe KM, Haass C. Emerging Microglia Biology Defines Novel Therapeutic Approaches for Alzheimer's Disease. *Neuron*. 2020;108(5):801-821. doi:10.1016/j.neuron.2020.09.029

14. Guerreiro R, Gibbons E, Tábuas-Pereira M, Kun-Rodrigues C, Santo GC, Bras J. Genetic architecture of common non-Alzheimer's disease dementias. *Neurobiol Dis*. 2020;142(May):104946. doi:10.1016/j.nbd.2020.104946

15. Scheltens P, De Strooper B, Kivipelto M, et al. Alzheimer's disease. *Lancet*. 2021;397(10284):1577-1590. doi:10.1016/S0140-6736(20)32205-4

16. Vatanabe IP, Manzine PR, Cominetti MR. Historic concepts of dementia and Alzheimer's disease: From ancient times to the present. *Rev Neurol (Paris)*. 2020;176(3):140-147. doi:10.1016/j.neurol.2019.03.004

17. McKeith IG, Dickson DW, Lowe J, et al. Diagnosis and management of dementia with Lewy bodies: Third report of the DLB consortium. *Neurology*. 2005;65(12):1863-1872. doi:10.1212/01.wnl.0000187889.17253.b1

18. McKeith I, Bradley F. B, Dickson DW, et al. Diagnosis and management of dementia with Lewy bodies: Fourth consensus report of the DLB Consortium. *Neurology*. 2017;89:88-100:1-14. https://www.ncbi.nlm.nih.gov/pubmed/28592453.

19. Savica R, Boeve BF, Logroscino G. *Epidemiology of Alpha-Synucleinopathies: From Parkinson Disease to Dementia with Lewy Bodies*. Vol 138. 1st ed. Elsevier B.V.; 2016. doi:10.1016/B978-0-12-802973-2.00009-4

20. Sezgin M, Bilgic B, Tinaz S, Emre M. Parkinson's Disease Dementia and Lewy Body Disease. *Semin Neurol*. 2019;39(2):274-282. doi:10.1055/s-0039-1678579

21. Borghammer P, Horsager J, Andersen K, et al. Neuropathological evidence of body-first vs. brain-first Lewy body disease. *Neurobiol Dis*. 2021;161(September):105557. doi:10.1016/j.nbd.2021.105557

22. Menšíková K, Matěj R, Colosimo C, et al. Lewy body disease or diseases with Lewy bodies? *npj Park Dis*. 2022;8(1). doi:10.1038/s41531-021-00273-9

23. Milán-Tomás Á, Fernández-Matarrubia M, Rodríguez-Oroz MC. Lewy body dementias: A coin with two sides? *Behav Sci (Basel)*. 2021;11(7):1-30. doi:10.3390/bs11070094

24. Jellinger KA. *Dementia with Lewy Bodies and Parkinson's Disease-Dementia: Current Concepts and Controversies*. Vol 125. Springer Vienna; 2018. doi:10.1007/s00702-017-1821-9

25. Hansen D, Ling H, Lashley T, Holton JL, Warner TT. Review: Clinical, neuropathological and genetic features of Lewy body dementias. *Neuropathol Appl Neurobiol*. 2019;45(7):635-654. doi:10.1111/nan.12554

26. Kalaitzakis ME, Pearce RKB, Gentleman SM. Clinical correlates of pathology in the claustrum in Parkinson's disease and dementia with Lewy bodies. *Neurosci Lett*. 2009;461(1):12-15. doi:10.1016/j.neulet.2009.05.083

27. Hansen D, Ling H, Lashley T, et al. Novel clinicopathological characteristics differentiate dementia with Lewy bodies from Parkinson's disease dementia. *Neuropathol Appl Neurobiol*. 2021;47(1):143-156. doi:10.1111/nan.12648

28. Tosto G, Monsell SE, Hawes SE, Bruno G, Mayeux R. Progression of extrapyramidal signs in Alzheimer's disease: Clinical and neuropathological correlates. *J Alzheimer's Dis*. 2015;49(4):1085-1093. doi:10.3233/JAD-150244

29. Yoo HS, Lee S, Chung SJ, et al. Clinical and striatal dopamine transporter predictors of β-Amyloid in dementia with Lewy bodies. *Neurology*. 2020;94(13):E1344-E1352. doi:10.1212/WNL.0000000000009168

30. Chung EJ, Babulal GM, Monsell SE, Cairns NJ, Roe CM, Morris JC. Clinical features of Alzheimer disease with and without Lewy bodies. *JAMA Neurol*. 2015;72(7):789-796. doi:10.1001/jamaneurol.2015.0606

31. Ferman TJ, Aoki N, Crook JE, et al. The limbic and neocortical contribution of α-synuclein, tau, and amyloid β to disease duration in dementia with Lewy bodies. *Alzheimer's Dement*. 2018;14(3):330-339. doi:10.1016/j.jalz.2017.09.014

32. Bellenguez C, Grenier-Boley B, Lambert JC. Genetics of Alzheimer's disease: where we are, and where we are going. *Curr Opin Neurobiol*. 2020;61:40-48. doi:10.1016/j.conb.2019.11.024

33. Bellenguez C, Küçükali F, Jansen IE, et al. New insights into the genetic etiology of Alzheimer's disease and related dementias. *Nat Genet*. 2022;54(4):412-436. doi:10.1038/s41588-022-01024-z

34. Noori A, Mezlini AM, Hyman BT, Serrano-Pozo A, Das S. Systematic review and meta-analysis of human transcriptomics reveals neuroinflammation, deficient energy metabolism, and proteostasis failure across neurodegeneration. *Neurobiol Dis*. 2021;149:105225. doi:10.1016/j.nbd.2020.105225

35. Felsky D, Roostaei T, Nho K, et al. Neuropathological correlates and genetic architecture of microglial activation in elderly human brain. *Nat Commun*. 2019;10(1):1-12. doi:10.1038/s41467-018-08279-3

36. Minett T, Classey J, Matthews FE, et al. Microglial immunophenotype in dementia with Alzheimer's pathology. *J Neuroinflammation*. 2016;13(1):1-10. doi:10.1186/s12974-016-0601-z

37. Salter MW, Stevens B. Microglia emerge as central players in brain disease. *Nat Med*. 2017;23(9):1018-1027. doi:10.1038/nm.4397

38. Chandra A, Valkimadi PE, Pagano G, Cousins O, Dervenoulas G, Politis M. Applications of amyloid, tau, and neuroinflammation PET imaging to Alzheimer's disease and mild cognitive impairment. *Hum Brain Mapp*. 2019;40(18):5424-5442. doi:10.1002/hbm.24782

39. Goedert M, Falcon B, Clavaguera F, Tolnay M. Prion-like mechanisms in the pathogenesis of tauopathies and synucleinopathies. *Curr Neurol Neurosci Rep*. 2014;14(11):1-11. doi:10.1007/s11910-014-0495-z

40. Thal DR, Rüb U, Orantes M, Braak H. Thal et al., 2002-Phases of Aß-deposition in the human brain and its relevance for the development of AD. 2002.

41. Leng F, Edison P. Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? *Nat Rev Neurol*. 2021;17(3):157-172. doi:10.1038/s41582-020-00435-y

42. Braak H, Del Tredici K, Rüb U, De Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging*. 2003;24(2):197-211. doi:10.1016/S0197-4580(02)00065-9

43. Talwar P, Kushwaha S, Chaturvedi M, Mahajan V. Systematic Review of Different Neuroimaging Correlates in Mild Cognitive Impairment and Alzheimer's Disease. *Clin Neuroradiol*. 2021;31(4):953-967. doi:10.1007/s00062-021-01057-7

44. Edison P, Archer HA, Gerhard A, et al. Microglia, amyloid, and cognition in Alzheimer's disease: An [11C](R)PK11195-PET and [11C]PIB-PET study. *Neurobiol Dis*. 2008;32(3):412-419. doi:10.1016/j.nbd.2008.08.001

45. Bradburn S, Murgatroyd C, Ray N. Neuroinflammation in mild cognitive impairment and Alzheimer's disease: A meta-analysis. *Ageing Res Rev*. 2019;50(November 2018):1-8. doi:10.1016/j.arr.2019.01.002

46. Fan Z, Brooks DJ, Okello A, Edison P. An early and late peak in microglial activation in Alzheimer's disease trajectory. *Brain*. 2017;140(3):792-803. doi:10.1093/brain/aww349

47. Hopperton KE, Mohammad D, Trépanier MO, Giuliano V, Bazinet RP. Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: A systematic review. *Mol Psychiatry*. 2018;23(2):177-198. doi:10.1038/mp.2017.246

48. Lier J, Streit WJ, Bechmann I. Beyond activation: Characterizing microglial functional phenotypes. *Cells*. 2021;10(9):1-13. doi:10.3390/cells10092236

49. Streit WJ, Xue QS. Microglia in dementia with Lewy bodies. *Brain Behav Immun*. 2016;55:191-201. doi:10.1016/j.bbi.2015.10.012

50. Olah M, Menon V, Habib N, et al. Single cell RNA sequencing of human microglia uncovers a subset associated with Alzheimer's disease. *Nat Commun*. 2020;11(1). doi:10.1038/s41467-020-19737-2

51. Serrano-Pozo A, Das S, Hyman BT. APOE and Alzheimer's disease: advances in genetics, pathophysiology, and therapeutic approaches. *Lancet Neurol*. 2021;20(1):68-80. doi:10.1016/S1474-4422(20)30412-9

52. Chia R, Sabir MS, Bandres-Ciga S, et al. Genome sequencing analysis identifies new loci associated with Lewy body dementia and provides insights into its genetic architecture. *Nat Genet*. 2021;53(3):294-303. doi:10.1038/s41588-021-00785-3

53. Nalls MA, Blauwendraat C, Vallerga CL, et al. Identification of novel risk loci, causal insights, and heritable risk for Parkinson's disease: a meta-analysis of genome-wide association studies. *Lancet Neurol*. 2019;18(12):1091-1102. doi:10.1016/S1474-4422(19)30320-5

54. Kaivola K, Shah Z, Chia R, International LBD Genomics Consortium, Scholz SW. Genetic evaluation of dementia with Lewy bodies implicates distinct disease subgroups. *Brain*. 2022;145(5):1757–1762. doi:10.1093/brain/awab402

55. Jin Y, Li F, Sonoustoun B, et al. APOE4 exacerbates α-synuclein seeding activity and contributes to neurotoxicity in Alzheimer's disease with Lewy body pathology. *Acta Neuropathol*. 2022;143(6):641-662. doi:10.1007/s00401-022-02421-8

56. Podleśny-Drabiniok A, Marcora E, Goate AM. Microglial Phagocytosis: A Disease-Associated Process Emerging from Alzheimer's Disease Genetics. *Trends Neurosci*. 2020;43(12):965-979. doi:10.1016/j.tins.2020.10.002

57. Gan-Or Z, Liong C, Alcalay RN. GBA-Associated Parkinson's Disease and Other Synucleinopathies Introduction—the Discovery of GBA Mutations in Parkinson's Disease. 2018. https://link.springer.com/content/pdf/10.1007%2Fs11910-018-0860-4.pdf.

58. Mullin S, Stokholm MG, Hughes D, et al. Brain Microglial Activation Increased in Glucocerebrosidase (GBA) Mutation Carriers without Parkinson's disease. *Mov Disord*. 2021;36(3):774-779. doi:10.1002/mds.28375

59. Tsuang D, Leverenz JB, Lopez OL, et al. GBA mutations increase risk for Lewy body disease with and without Alzheimer disease pathology. *Neurology*. 2012;79(19):1944-1950. doi:10.1212/WNL.0b013e3182735e9a

60. Feleke R, Reynolds RH, Smith AM, et al. Cross-platform transcriptional profiling identifies common and distinct molecular pathologies in Lewy body diseases. *Acta Neuropathol*. 2021;142(3):449-474. doi:10.1007/s00401-021-02343-x

61. Jinn S, Drolet RE, Cramer PE, et al. TMEM175 deficiency impairs lysosomal and mitochondrial function and increases α-synuclein aggregation. *Proc Natl Acad Sci U S A*. 2017;114(9):2389-2394. doi:10.1073/pnas.1616332114

62. Orme T, Hernandez D, Ross OA, et al. Analysis of neurodegenerative disease-causing genes in dementia with Lewy bodies. *Acta Neuropathol Commun*. 2020;8(1):1-11. doi:10.1186/s40478-020-0879-z

63. Iannaccone S, Cerami C, Alessio M, et al. In vivo microglia activation in very early dementia with Lewy bodies, comparison with Parkinson's disease. *Park Relat Disord*. 2013;19(1):47-52. doi:10.1016/j.parkreldis.2012.07.002

64. Surendranathan A, Su L, Mak E, et al. Early microglial activation and peripheral inflammation in dementia with Lewy bodies. *Brain*. 2018;141(12):3415-3427. doi:10.1093/brain/awy265

65. Edison P, Ahmed I, Fan Z, et al. Microglia, amyloid, and glucose metabolism in parkinson's disease with and without dementia. *Neuropsychopharmacology*. 2013;38(6):938-949. doi:10.1038/npp.2012.255

66. Gao P, Zhang F. Neuroinflammation in Parkinson's disease: a meta-analysis of PET imaging studies. *J Neurol*. 2022;(269):2304–2314. https://doi.org/10.1007/s00415-021-10877-z.

67. Badanjak K, Fixemer S, Smajić S, Skupin A, Grünewald A. The contribution of microglia to neuroinflammation in parkinson's disease. *Int J Mol Sci*. 2021;22(9). doi:10.3390/ijms22094676

68. Kouli A, Camacho M, Allinson K, Gray CHW. Neuroinflammation and protein pathology in Parkinson ' s disease dementia. 2020:1-19.

69. Imamura K, Hishikawa N, Sawada M, Nagatsu T, Yoshida M, Hashizume Y. Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. *Acta Neuropathol*. 2003;106(6):518-526. doi:10.1007/s00401-003-0766-2

70. Croisier E, Moran LB, Dexter DT, Pearce RKB, Graeber MB. Microglial inflammation in the parkinsonian substantia nigra: Relationship to alpha-synuclein deposition. *J Neuroinflammation*. 2005;2:1-8. doi:10.1186/1742-2094-2-14

71. Doorn KJ, Moors T, Drukarch B, van de Berg WDJ, Lucassen PJ, van Dam AM. Microglial phenotypes and toll-like receptor 2 in the substantia nigra and hippocampus of incidental Lewy body disease cases and Parkinson's disease patients. *Acta Neuropathol Commun*. 2014;2(1):1-17. doi:10.1186/s40478-014-0090-1

72. Smajić S, Prada-Medina CA, Landoulsi Z, et al. Single-cell sequencing of human midbrain reveals glial activation and a Parkinson-specific neuronal state. *Brain*. 2022;145(3):964-978. doi:10.1093/brain/awab446

73. Weiss F, Labrador-Garrido A, Dzamko N, Halliday G. Immune responses in the Parkinson's disease brain. *Neurobiol Dis*.

2022;168(January):105700. doi:10.1016/j.nbd.2022.105700

74. Ray WJ, Buggia-Prevot V. Novel Targets for Alzheimer's Disease: A View beyond Amyloid. *Annu Rev Med*. 2021;72:15-28. doi:10.1146/annurev-med-052919-120219

75. Mukhopadhyay S, Banerjee D, Kosagisharaf JR. A Primer on the Evolution of Aducanumab: The First Antibody Approved for Treatment of Alzheimer's Disease. *J Alzheimer's Dis*. 2021;83(4):1537-1552. doi:10.3233/JAD-215065

76. Decourt B, Boumelhem F, Pope ED, Shi J, Mari Z, Sabbagh MN. Critical Appraisal of Amyloid Lowering Agents in AD. *Curr Neurol Neurosci Rep*. 2021;21(8). doi:10.1007/s11910-021-01125-y

77. Lee G, Cummings J, Decourt B, Leverenz JB, Sabbagh MN. Clinical drug development for dementia with Lewy bodies: past and present. *Expert Opin Investig Drugs*. 2019;28(11):951-965. doi:10.1080/13543784.2019.1681398

78. Montine TJ, Phelps CH, Beach TG, et al. National institute on aging-Alzheimer's association guidelines for the neuropathologic assessment of Alzheimer's disease: A practical approach. *Acta Neuropathol*. 2012;123(1):1-11. doi:10.1007/s00401-011-0910-3

79. Braak H, Braak E. Neuropathological Stageing of Alzheimer-related changes. *Acta Neuropathol*. 1991;82:239-259.

80. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: A clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry*. 1992;55(3):181-184. doi:10.1136/jnnp.55.3.181

81. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders DSM-V. 5th ed. 2014:720–63.

82. Josephs KA, Murray ME, Whitwell JL, et al. Updated TDP-43 in Alzheimer's disease staging scheme. *Acta Neuropathol*. 2016;(131):571–585. doi:https://doi.org/10.1007/s00401-016-1537-1

83. Eloy C, Vale J, Curado M, et al. Digital pathology workflow implementation at ipatimup. *Diagnostics*. 2021;11(11):1-11. doi:10.3390/diagnostics11112111

84. Humphries MP, Maxwell P, Salto-Tellez M. QuPath: The global impact of an open source digital pathology system. *Comput Struct Biotechnol J*. 2021;19:852-859. doi:10.1016/j.csbj.2021.01.022

85. Irwin DJ, Grossman M, Weintraub D, et al. Neuropathological and genetic correlates of survival and dementia onset in synucleinopathies: a retrospective analysis. *Lancet Neurol*. 2017;16(1):55. doi:10.1016/S1474-4422(16)30291-5

86. Bethlehem RAI, Seidlitz J, White SR, et al. Brain charts for the human lifespan. *Nature*. 2022;604(7906):525-533. doi:10.1038/s41586-022-04554-y

87. Santana I, Farinha F, Freitas S, Rodrigues V, Carvalho Á. The epidemiology of dementia and Alzheimer disease in Portugal: Estimations of prevalence and treatment-costs. *Acta Med Port*. 2015;28(2):182-188. doi:10.20344/amp.6025

88. Agosta F, Galantucci S, Filippi M. Advanced magnetic resonance imaging of neurodegenerative diseases. *Neurol Sci*. 2017;38(1):41-51. doi:10.1007/s10072-016-2764-x

89. Shepherd CE, Thiel E, McCann H, Harding AJ, Halliday GM. Cortical inflammation in Alzheimer disease but not dementia with Lewy bodies. *Arch Neurol*. 2000;57(6):817-822. doi:10.1001/archneur.57.6.817

90. Attems J, Toledo JB, Walker L, et al. Neuropathological consensus criteria for the evaluation of Lewy pathology in post-mortem brains: a multi-centre study. *Acta Neuropathol*. 2021;141(2):159-172. doi:10.1007/s00401-020-02255-2

91. Homma T, Toru S, Hirokawa K, Uchihara T. Digital mapping of Lewy bodies and neurites in alpha-synuclein stained large cerebral hemispheric sections from three patients with dementia with Lewy bodies showing psychotic manifestations: A pilot study. *Neurosci Lett*. 2021;747(January):135708. doi:10.1016/j.neulet.2021.135708

92. Boillée S. Local and remote interactions between macrophages and microglia in neurological conditions. *Curr Opin Immunol*. 2022;(74):118-124. doi:10.1016/j.coi.2021.11.006

93. Guerrero A, De Strooper B, Arancibia-Cárcamo IL. Cellular senescence at the crossroads of inflammation and Alzheimer's disease. *Trends Neurosci*. 2021;44(9):714-727. doi:10.1016/j.tins.2021.06.007

94. Matsudaira T, Prinz M. Life and death of microglia: mechanisms governing microglial states and fates. *Immunol Lett*. 2022;245(January):51-60. doi:10.1016/j.imlet.2022.04.001

95. Mathys H, Davila-Velderrain J, Peng Z, et al. Single-cell transcriptomic analysis of Alzheimer's disease. *Nature*. 2019;570(7761):332-337. doi:10.1038/s41586-019-1195-2

96. Thrupp N, Sala Frigerio C, Wolfs L, et al. Single-Nucleus RNA-Seq Is Not Suitable for Detection of Microglial Activation Genes in Humans. *Cell Rep*. 2020;32(13):108189. doi:10.1016/j.celrep.2020.108189

U.PORTO

M ICBAS **2022**

Neuroinflammation patterns in Alzheimer´s disease, Dementia with Lewy bodies and cases with mixed pathology

António Gil Cabral de Azevedo

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

