# University of Southampton

Faculty of Medicine

Clinical and Experimental Sciences


# Inflammation in Dementia with Lewy bodies and Alzheimer's disease


by

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Thesis for the degree of Doctor of Philosophy

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## Abstract

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Dementia with Lewy bodies (DLB) is the second most common neurodegenerative cause of dementia. However, the aetiology of DLB remains poorly understood in comparison with Alzheimer's disease (AD) and Parkinson's disease (PD). Current evidence supports that neuroinflammation, with involvement of the peripheral immune system, occurs in both AD and PD. Genetic studies in particular support an aetiological role for inflammation in AD rather than it being merely a consequence of neurodegeneration.

Despite extensive research into the role of inflammation in AD and PD, there have been a paucity of studies in DLB. I hypothesised that DLB would show a specific cerebral and systemic inflammatory profile. In order to investigate this hypothesis, two studies were performed. A cross-sectional clinical study investigated peripheral inflammation in DLB, AD and controls using flow cytometry and multiplex immunoassay, and post-mortem human brain tissue work examined microglial immunophenotype in DLB, AD and controls using immunohistochemistry.

The clinical study revealed increased serum concentrations of two pro-inflammatory cytokines (IL1β and IL6) in DLB compared with controls. In addition, flow cytometry showed a decline in cell populations associated with adaptive immunity (helper T cells and activated B cells) in DLB compared to AD. These data demonstrate senescence of the adaptive immune system in DLB compared with AD, possibly driving a chronic inflammatory state.

The post-mortem work confirmed increased cerebral protein deposition in DLB and AD, but the two diseases showed markedly different microglial phenotypes. AD was characterised by a strong phagocytic microglial phenotype, but in DLB there was no evidence of increased activation of any phenotype. These findings may be associated with the different profiles of the peripheral adaptive immune system, with AD characterised by increased antibody-mediated microglial activation compared with DLB.

The two studies undertaken as part of this project appear to show that the immunophenotype of DLB is distinct from that of AD, with cerebral inflammation not a primary feature of DLB as it is in AD. This has therapeutic implications in that the use of anti-inflammatory therapy may not indicated in DLB. Furthermore, identification of a unique peripheral immune profile in DLB warrants further exploration in order to develop a blood-based immune biomarker that could differentiate these two diseases.

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# Declaration of Authorship

I, **Jay Amin**, declare that this thesis, entitled **"Inflammation in Dementia with Lewy bodies and Alzheimer's disease"**, and the work presented in it, are my own and have been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been presented at conferences and meetings (see Appendix A for further details) and published as:
    - Jay Amin, Anthony Williams, Jessica Teeling, Robert Dorey, Daisy Williams, Emmanuele Tommasino, Delphine Boche, Clive Holmes. Central and systemic inflammation in Dementia with Lewy bodies and Alzheimer's disease [abstract]. Neuropathology and Applied Neurobiology 2017; 43(supplementary issue 1):12-13
    - Jay Amin, Anthony Williams, Jessica Teeling, Delphine Boche, Clive Holmes. Central and systemic inflammation in Dementia with Lewy bodies and Alzheimer's disease [abstract]. Neuropathology and Applied Neurobiology 2016; 42(supplementary issue 1):36
    - Jay Amin, Anthony Williams, Jessica Teeling, Delphine Boche, Clive Holmes Central and systemic inflammation in Dementia with Lewy bodies and Alzheimer's disease [abstract]. American Journal of Neurodegenerative Disease 2015; 4(supplementary issue 1):64. www.ajnd.us/ISSN:2165-591X/2015 International DLB Conf.

Signed:.....

Date: .....

# List of Accompanying Materials

1. Bona fide researchers, subject to registration may request supporting data via University of Southampton repository: https://doi.org/10.5258/SOTON/D1019

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Lastly, this work would not have been completed without the love and support of my wife Varsha. However, it is very likely that it would have been completed significantly earlier without the interference of our daughter Arya!

# Contribution

I successfully applied for a Clinical Research Fellowship from Alzheimer's Research UK for £213,317.40 with guidance from Prof Clive Holmes and Prof Delphine Boche.

For the clinical study, I developed the study materials and protocol, and applied for regulatory approvals under supervision from Prof Holmes. I set up on-site procedures for isolating peripheral blood mononuclear cells. I trained staff at both study sites and undertook monitoring visits. I performed all screening and recruitment for the main site and provided verification for recruitment at the secondary site. In addition, at the main site, I undertook all but one of the study visits myself, including consenting, administrating rating scales and blood processing. Optimisation of flow cytometry and stimulation experiments was performed under the supervision of Dr Lindsey Chudley and Dr Yifang Gao, but I performed all experiments. I assisted Dr Laurie Lau in performing cytokine analysis. Data entry for the clinical study was undertaken by medical student Flo Smith, under my supervision.

For the post-mortem study, I secured suitable brain tissue from registered brain banks, with guidance from Prof Boche. Many protocols using antibodies against markers of inflammation and neuropathology had already been optimised for use in immunohistochemistry in the Boche laboratory. Immunostaining for α-syn was performed by the Cellular Pathology Department at University Hospitals Southampton, and immunostaining for Aβ, ptau and Iba1 was performed by Jenny Norman in the Histochemistry Research Unit at the University of Southampton. All other experiments were performed or supervised by me. Medical students who I trained and supervised were: Robert Dorey, Daisy Williams, Emanuele Tommasino and Yuri Casal. Digital images of immunostained tissue were taken and processed either by me or the students listed above.

I performed all data analysis presented in this thesis, with guidance and supervision from Scott Harris (medical statistician), Prof Holmes and Prof Boche. This thesis was written solely by me, following comments received from my supervisors.

# Abbreviations

| | |
|---|---|
| **α-syn** | Alpha-synuclein |
| **ABC** | Avidin-biotin complex |
| **AD** | Alzheimer's disease |
| **APOE** | Apolipoprotein E |
| **APP** | Amyloid precursor protein |
| **Aβ** | Amyloid-beta |
| **BA** | Brodmann area |
| **BAP** | British association of psychopharmacologists |
| **BBB** | Blood-brain barrier |
| **CAF** | Clinician assessment of fluctuation |
| **CCL** | Chemokine ligand |
| **CCR7** | C-C chemokine receptor type 7 |
| **CD** | Cluster of differentiation |
| **ChI** | Cholinesterase inhibitor |
| **CHI3L1** | Chitinase-3-like-1 |
| **CIDL** | Cerebral inflammation in Dementia with Lewy bodies |
| **CNS** | Central nervous system |
| **CRP** | C-reactive protein |
| **CSDD** | Cornell scale for depression in dementia |
| **CSF** | Cerebrospinal fluid |
| **CT** | Computerised tomography |
| **DAB** | Diaminobenzidine |
| **DaT** | Dopamine active transporter |
| **DLB** | Dementia with Lewy bodies |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ERGO** | Ethics and research governance online |
| **FACS** | Fluorescence activated cell sorting |
| **FcγR** | Fc gamma receptor |
| **FCSRT-IR** | Free and cued selective reminding test – immediate recall |

| | |
|---|---|
| **FFPE** | Formalin-fixed paraffin-embedded |
| **FMO** | Fluorescence-minus-one |
| **FTD** | Frontotemporal dementia |
| **GBA** | Glucocerebrosidase-A |
| **GFAP** | Glial fibrillary acidic protein |
| **GP** | General practice |
| **GWAS** | Genome-wide association study |
| **HLA-DR** | Human leukocyte antigen – antigen D related |
| **HRU** | Histochemistry research unit |
| **Iba1** | Ionized Calcium-binding adapter molecule 1 |
| **ICD** | International classification of diseases |
| **IFN** | Interferon |
| **Ig** | Immunoglobulin |
| **IgG** | Immunoglobulin G |
| **IL** | Interleukin |
| **JDR** | Join dementia research |
| **LB** | Lewy body |
| **LBD** | Lewy body disease |
| **LLOD** | Lower limit of detection |
| **LN** | Lewy neurite |
| **LNDBB** | London neurodegenerative disease brain bank |
| **LPS** | Lipopolysaccharide |
| **LRP** | Lewy-related pathology |
| **LRRK2** | Leucine-rich repeat kinase 2 |
| **MARC** | Memory assessment and research centre |
| **MCI** | Mild cognitive impairment |
| **MHC-II** | Major histocompatibility complex class II |
| **MMSE** | Mini mental state examination |
| **MoCA** | Montreal cognitive assessment |
| **MRC** | Medical research council |
| **MRI** | Magnetic resonance imaging |
| **MSD** | Meso Scale Discovery |

| | |
|---|---|
| **MSR-A** | Macrophage scavenger receptor-A |
| **NFT** | Neurofibrillary tangles |
| **NIA-AA** | National institute on aging – Alzheimer's association |
| **NMDA** | N-methyl-D-aspartate |
| **NPI** | Neuropsychiatric inventory |
| **NREC** | NHS research ethics committee |
| **NSAID** | Non-steroidal anti-inflammatory drug |
| **PBMC** | Peripheral blood mononuclear cells |
| **PBS** | Phosphate buffered saline |
| **PD** | Parkinson's disease |
| **PDD** | Parkinson's disease dementia |
| **PET** | Positron emission tomography |
| **PHA** | Phytohaemagglutinin |
| **PIS** | Participant information sheet |
| **PMD** | Post-mortem delay |
| **ptau** | Hyperphosphorylated tau |
| **R&D** | Research and development |
| **REM** | Rapid eye movement |
| **RGB** | Red-green-blue |
| **RNA** | Ribonucleic acid |
| **SCARB2** | Scavenger receptor B2 |
| **SILAD** | Systemic inflammation in Dementia with Lewy bodies and Alzheimer's disease |
| **SNCA** | Synuclein alpha (gene) |
| **SPECT** | Single photon emission computed tomography |
| **SWDBB** | South West dementia brain bank |
| **TEMRA** | Terminally differentiated effector memory cells re-expressing CD45RA |
| **TNF** | Tumour necrosis factor |
| **TREM2** | Triggering receptor expressed on myeloid cells 2 |
| **TRIS** | Trisaminomethane buffered saline |
| **TSPO** | Translocator protein |
| **UPDRS** | Unified Parkinson's disease rating scale |

| | |
|---|---|
| **VaD** | Vascular dementia |
| **WISH** | Wessex investigational sciences hub |

# Chapter 1: Introduction

Dementia is a condition that places a huge burden upon society and afflicts an estimated 670,000 people in the United Kingdom (UK) and 35.6 million people globally, with that figure set to double every 20 years [1, 2]. It has also been approximated that the total cost of dementia to UK society is £26 billion per annum, made up of health and social care costs, as well as costs contributed by unpaid carers [3]. Furthermore, the worldwide costs of dementia were estimated to be a staggering US$604 billion in 2010 [4].

The prevention and management of dementia is one of the greatest public health challenges in our generation [5]. Despite several drug treatments being licenced, there are still no disease-modifying therapies available. Indeed, the prevention and treatment of dementia, along with greater investment in dementia research, was agreed as a priority for national governments at the 2013 G8 summit hosted by the UK.

The term dementia can be defined as a group of cognitive or neuropsychiatric symptoms that cause functional impairment, and that are not explained by delirium or any other major psychiatric disorder [6]. It can be thought of as a diagnosis of exclusion, with alternative causes of cognitive impairment such as stroke or electrolyte imbalance having to be discounted before dementia can be considered. Symptoms of dementia can include memory impairment, poor judgement, impaired visuospatial ability, impaired language and behavioural change. According to the International Classification of Diseases (ICD) criteria, cognitive impairment must be present for at least six months for a diagnosis of dementia [7]. The nature of onset and pattern of cognitive deficits can often provide pointers as to the cause of dementia.

There are numerous known causes of dementia. The most common cause is Alzheimer's disease (AD), making up approximately two thirds of all cases [8]. Other major sub-types of dementia include: Vascular dementia (VaD), Dementia with Lewy bodies (DLB), Frontotemporal dementia (FTD) and Parkinson's disease dementia (PDD).

## 1.1 Dementia with Lewy bodies

The term Lewy body disease (LBD) encompasses Dementia with Lewy bodies (DLB), Parkinson's disease (PD) and Parkinson's disease dementia (PDD) [9], with all three diseases sharing Lewy bodies as their common brain pathology. In DLB and PDD there are prominent cognitive symptoms, whereas PD involves primarily motor, but also non-motor, symptoms [10]. It has been shown that the majority of PD patients go on to develop PDD 10 years after the onset of motor symptoms [11, 12]. Figure 1.1 illustrates the nomenclature of these three Lewy body diseases.

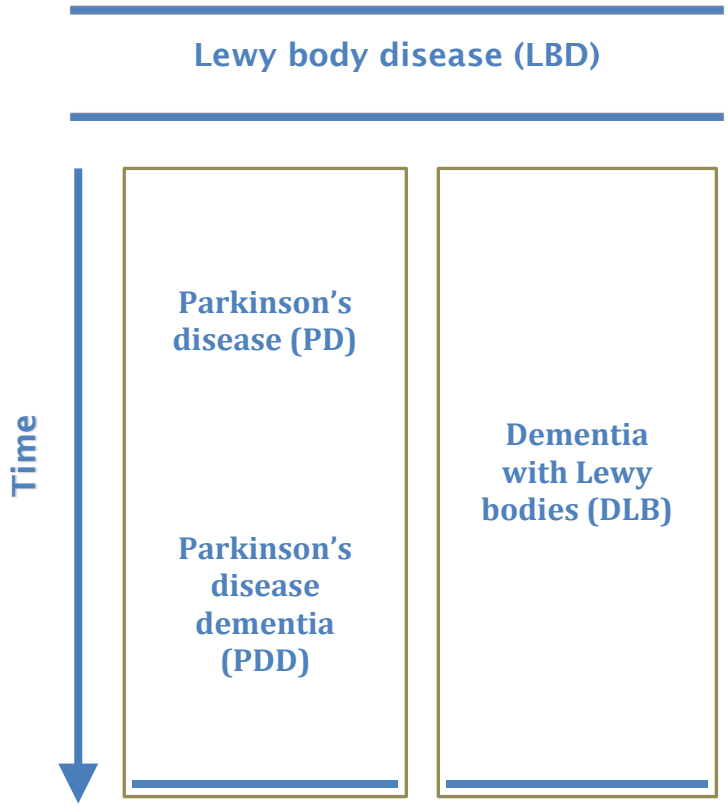


**Figure 1.1: Classification of Lewy body diseases**
Dementia with Lewy bodies (DLB) is diagnosed when the onset of dementia is before the onset of Parkinsonism or within one year afterwards. Parkinson's disease dementia (PDD) is diagnosed when the onset of dementia is greater than one year after the onset of Parkinsonism.

### 1.1.1 History

DLB is named after the American-German neurologist Friedrich Lewy, who in 1912 first described intra-cytoplasmic inclusion bodies in the brain of a case of

paralysis agitans [13]. Many decades later in 1972, the Japanese psychiatrist Kenji Kosaka confirmed the presence of Lewy bodies in the cortex of a patient with atypical AD who also had motor symptoms [14]. However, it was not until 1992 when the neuropathological findings of Lewy bodies were connected with a syndrome of clinical symptoms that is now thought of as DLB [15]. Four international consensus criteria have been published since then [9, 16-18], providing updated guidance on the clinical and neuropathological diagnosis of the disease. Furthermore, in 2013 DLB was included in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders [19].

### 1.1.2 Epidemiology

DLB is the second most common neurodegenerative cause of dementia, behind AD [20]. A recent meta-analysis concluded that DLB has a UK prevalence rate of 4.2% of cases of dementia in the community and 7.5% in secondary care [21]. The same study estimated that the annual incidence rate for DLB was 3.8% of new dementia diagnoses [21]. However, there does appear to be some geographical variation in the prevalence rate of DLB in the UK, most likely due to differences in clinician awareness and diagnostic practice [22].

Despite improvements in the diagnosis rate of DLB since the third international consensus criteria were published over 10 years ago [21], there is still evidence that DLB is often misdiagnosed as AD or PD [23]. Therefore, it is likely that the actual prevalence of DLB continues to be underestimated and is in fact significantly higher than indicated by the values presented above [24]. However, work is underway in the UK to develop a toolkit to improve detection of LBDs by clinicians [25].

### 1.1.3 Aetiology

The underlying cause of DLB remains unknown. Some of the current aetiological theories stem from studies in DLB, but most have been extrapolated from studies in PD and AD.

Numerous studies have shown familial aggregation of the core clinical features of DLB, or of DLB itself [26-29]. However, a study examining concordance rates between twins, one of whom had post-mortem confirmed DLB, showed no

genetic concordance for DLB [30]. The sample size in this trial was small and it also included mixed AD and DLB cases, perhaps impeding the detection of a significant genetic concordance for DLB. Nevertheless, the heritability of DLB has been estimated to be as high as 40% [31], suggesting a significant genetic component to the aetiology of the disease.

Genetic studies have revealed that genes associated with increased risk of AD (Apolipoprotein E - *APOE*) and PD (synuclein alpha – *SNCA*, and scavenger receptor B2 - *SCARB2*) are also associated with increased risk of sporadic DLB [32]. Since specific *APOE* alleles are associated with AD, which is a disease characterised by deposition of amyloid-beta (Aβ), it is likely that this gene also plays a role in the pathogenic process in DLB. *SNCA* is a gene that codes for the protein alpha-synuclein (α-syn), a fundamental component of neuropathology in LBDs. Although exceedingly rare, mutations in the *SNCA* gene have been shown to be associated with familial PD [33], familial PDD [34] and DLB [35]. In addition, mutations in the gene coding for lysosomal enzyme Glucocerebrosidase-A 1 (*GBA1*) have also been shown to be a significant risk factor for both DLB and PD [36]. Both *GBA1* and *SCARB2* are known to be associated with the lysosomal membrane, suggesting a role in DLB for altered processing of phagocytosed material. Indeed, lysosomal depletion has been found in PD brains as well as in experimental models of PD [37]. The genetic polymorphisms described above support the hypothesis that aggregation of α-syn, in tandem with lysosomal dysfunction, could be key players in protein aggregation, neuronal cell death and in the overall aetiology of DLB.

Recently a large multi-centre genome-wide association study (GWAS) examined the genotypes of 1,743 DLB patients of European ancestry and confirmed previous loci for *APOE*, *SNCA* and *GBA1* [38]. Meanwhile, a genome-wide analysis of genetic correlations quantified the genetic overlap of DLB with AD, and with PD. It showed that, when comparing these diseases for genetic correlation, DLB shares approximately the same amount of genetic risk determinants with AD and PD [39]. *APOE* genotype has been shown to be the strongest genetic risk factor for DLB and this remains even after restricting analysis to neuropathologically confirmed cases [32, 38]. Indeed, genotyping of a large cohort of well-defined neuropathologically confirmed cases reveals that *APOE* ε4 carriers were strongly associated with DLB (odds ratio 6.1), but

even more strongly associated with AD (odds ratio 9.9) [40]. Interestingly this study also showed that the strongest association for ε4 carriers was in cases diagnosed as mixed AD and DLB.

Several studies have demonstrated significant deficit of the neurotransmitter acetylcholine in DLB and PDD brains, even more pronounced than found in AD [41-43]. Cholinergic deficit in DLB is thought to be prominent from the very early stages of the disease and supports the use of acetylcholinesterase inhibitors as treatments in DLB.

The significance of abnormal intracellular accumulations of α-syn, in the form of Lewy bodies (LB), remains uncertain. It is unclear whether LB are: a) the trigger for pathogenesis and neurodegeneration, b) markers of neuronal injury, c) represent a protective neuronal response, d) simply an epiphenomenon, or e) a combination of these factors. α-syn is a 140 amino-acid protein that is highly abundant in the brain and located in the presynaptic neuronal terminal [44]. The physiological role of α-syn is thought to be related to neurotransmitter release through regulation of synaptic vesicle recycling [45], linking impairment of this role to the cholinergic deficit in DLB described above. The exact role of α-syn pathology in the aetiology and pathogenesis of DLB remains unclear, but it is interesting to note that aberrant protein accumulation in AD has been proposed to be a physiological neuroprotective response, rather than a harbinger of disease [46].

The observation that α-syn can propagate through the brain by a prion-like mechanism [47] has introduced a novel aetiological factor for the development and progression of PD. The spread of pathology through sequential transfer of proteins from one brain cell to another has been implicated in a number of neurodegenerative diseases [48]. Specifically in PD, Braak et al. proposed that aggregation of α-syn in the brainstem and olfactory bulb were typical early features of the disease [49], with pathology spreading in a largely predictable topographical manner through the limbic system and then to the neocortex. Furthermore, there is evidence that α-syn pathology has been found in the enteric nervous system and colonic submucosa in PD, prior to motor symptoms emerging, possibly reaching the brainstem via the vagus nerve [50]. This uncovers the possibility that gut entry of α-syn pathology from the environment may be a possible further aetiological factor for PD. However, a

recent article has thoroughly reviewed evidence in this area over the last decade and concluded that there are at least as many arguments against this theory as for it, including a lack of reproducible results and a lack of confirmation in large autopsy cohorts [51]. It is also not established as to whether the same aetiological theory may apply to DLB.

The aetiology of PD itself has been the subject of much debate. Numerous environmental and dietary risk factors have been proposed as risk factors for developing sporadic PD, including pesticides, herbicides and exposure to heavy metals, with further protective factors identified including smoking and caffeine [52]. Above all, age is the major known risk factor for developing PD [53]. Alterations in cerebral and peripheral inflammation have also been proposed as aetiological factors in DLB and PD [54], as discussed further in section 1.6.

### 1.1.4 Clinical features and diagnosis

The latest international consensus criteria for the diagnosis of DLB were published in 2017 and included updated guidance on both clinical and neuropathological diagnosis [18].

The clinical diagnostic criteria for DLB are summarised in table 1.1. A diagnosis of dementia is mandatory, with prominent memory impairment not necessarily occurring in the early stages of the disease. Deficits on tests of attention, executive function and visuoperceptual ability may be more prominent in DLB [18, 55]. Probable DLB can be diagnosed clinically with the presence of two or more core clinical features, with or without the presence of indicative biomarkers; or with at least one core clinical feature and at least one indicative biomarker.

The four core clinical features of DLB are: fluctuating cognition, recurrent visual hallucinations, spontaneous motor features of Parkinsonism and rapid eye movement (REM) sleep behaviour disorder. Fluctuations in DLB occur as spontaneous alterations in cognition, alertness and attention. Visual hallucinations in DLB are typically complex, recurrent and well-formed, affecting up to 80% of DLB patients. Spontaneous motor features of Parkinsonism that commonly occur in DLB include bradykinesia, tremor and

rigidity. REM sleep behaviour disorder is a parasomnia that classically presents with patients sleep-talking or acting out their dreams due to lack of normal REM sleep atonia [18]. Supportive clinical features are also shown in table 1.1 and although they are commonly present in DLB, they lack diagnostic specificity [18].

Indicative biomarkers for DLB include reduced dopamine active transporter (DaT) uptake in the basal ganglia on Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT), low uptake on $^{123}$I-metaiodobenzylguanidine (MIBG) myocardial scintigraphy and polysomnographic confirmation of REM sleep without atonia [18].

The use of DaT imaging in DLB is now well-established. The most common ligand used to image the density of presynaptic DaT is N-fluoropropyl-2β-carbomethoxy-3β-(4-iodophenyl)nortropane ($^{123}$I-FP-CIT). Clinical studies have shown high sensitivity (77-94%) and high specificity (88-97%) for DLB [56, 57]. It has also been shown that $^{123}$I-FP-CIT imaging increases clinicians' diagnostic certainty in dementia, principally when the scan result is positive [58].

$^{123}$I-MIBG is a noradrenaline analogue used in cardiac scintigraphy to determine the loss of sympathetic cardiac innervation, and has been shown to differentiate DLB from AD with a sensitivity of 69% and specificity of 87% [59]. The latest National Institute for Health and Care Excellence (NICE) guidance on the assessment and management of Dementia recommends use of $^{123}$I-FP-CIT in cases where the diagnosis of DLB is uncertain, and further recommends use of $^{123}$I-MIBG when $^{123}$I-FP-CIT is unavailable [60].

REM sleep without atonia, which is confirmed using polysomnography, manifests clinically with REM sleep behaviour disorder. The combination of this clinical feature and biomarker confirmation in patients with dementia has been shown to be associated with a >90% likelihood of a synucleinopathy like DLB [61].

**Table 1.1: Consensus criteria for clinical diagnosis of DLB**

| | |
|---|---|
| **Dementia** | • Dementia is defined as a progressive decline in cognition of sufficient magnitude to interfere with normal social or occupational functions. |
| **Core clinical features** | • Fluctuating cognition with pronounced variations in attention and alertness.<br>• Recurrent visual hallucinations that are typically well-formed and detailed<br>• Spontaneous motor features of Parkinsonism<br>• REM sleep behaviour disorder |
| **Supportive clinical features** | • Severe sensitivity to antipsychotic agents, postural instability, repeated falls, transient episodes of unresponsiveness, severe autonomic dysfunction, hypersomnia, hyposmia, hallucinations in other modalities, systematised delusions, apathy, anxiety, depression |
| **Indicative biomarkers** | • Reduced dopamine transporter uptake in basal ganglia demonstrated by SPECT or PET<br>• Abnormal $^{123}$iodine-MIBG myocardial scintigraphy<br>• Polysomnographic confirmation of REM sleep without atonia |
| **Supportive biomarkers** | • Relative preservation of medial temporal lobe structures on CT/MRI scan<br>• Generalised low uptake on SPECT/PET perfusion scan with reduced occipital activity<br>• Prominent slow wave activity of EEG with temporal lobe transient sharp waves |

Adapted from McKeith et al. 2017 [18]

### 1.1.5 Neuropathology

The neuropathology of DLB is defined by the presence of Lewy bodies (LB) in the cerebrum [9]. LB are insoluble protein aggregates made up primarily of α-syn [62]. There are several other pathological features of DLB, namely Lewy neurites (LN), neuronal loss and synaptic loss. LB and LN can be described together as Lewy-related pathology (LRP) and are illustrated in figure 1.2. LRP is also the hallmark pathological feature of both PD and PDD, and has been shown to spread sequentially from the brainstem via the midbrain and into the neocortex in PD [63]. The distribution of LRP pathology in mixed DLB and AD

cases may differ however, with LRP pathology possibly originating from the olfactory bulb and progressing to the limbic system but without significant brainstem involvement [64].

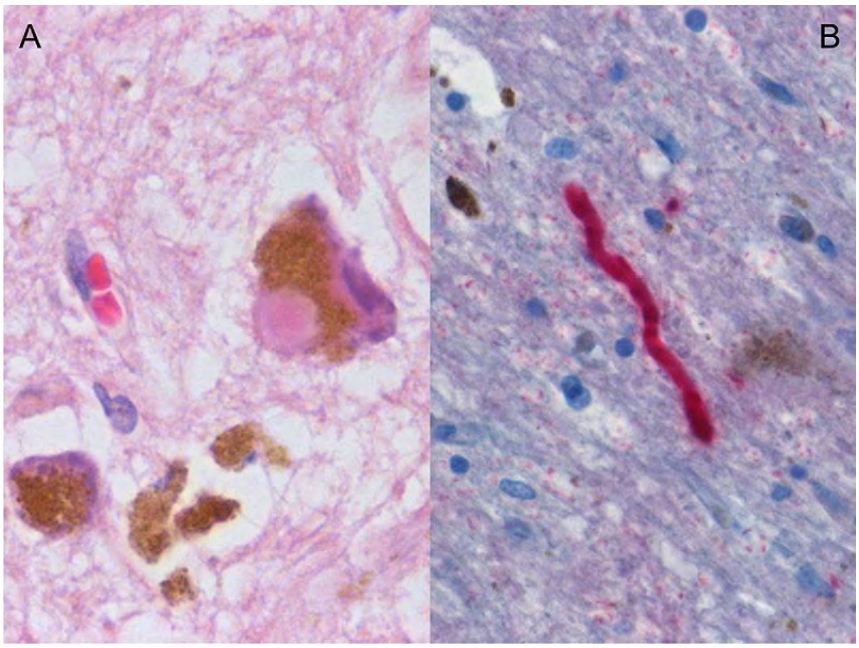


**Figure 1.2: Lewy-related pathology**
Images from substantia nigra in an individual with Parkinson's disease, immunostained for haematoxylin and eosin (A, x500) and alpha-synuclein (B, x400). Intracellular Lewy body seen in (A) and Lewy neurite seen in (B). Image taken from Werner et al. 2008 [65].

The 2017 international consensus criteria for the post-mortem neuropathological diagnosis of DLB is based upon the severity of LRP being assessed concurrently with the level of AD pathology. The semi-quantitative categorisation of LRP severity into mild, moderate, severe and very severe remains from the previous consensus criteria [9]. Both LRP and hyperphosphorylated tau (ptau) Braak stages are assigned to samples taken from the brainstem, limbic and neocortical regions. The brain regions used for sampling are based on the National Institute of Aging (NIA)-Reagan criteria used for diagnosing AD [66]. A likelihood ratio is then assigned to a case where the neuropathologist concludes to what extent the observed LRP explains the DLB clinical syndrome of that patient. A diffuse neocortical distribution of LRP with a low or intermediate ptau Braak stage (0-4) would be most likely to explain a DLB clinical syndrome, whereas a brainstem or

amygdala predominant distribution of LRP with any ptau Braak stage (0-6) would be least likely to explain a DLB clinical syndrome [9].

AD pathology in the form of Aβ plaques and ptau neurofibrillary tangles (NFT), are also frequently present in DLB, although the extent of ptau pathology has been shown to be lower in DLB compared to AD [67-69]. Howlett et al. in particular showed that combined LRP and AD pathology, particularly in cortical region Brodmann area (BA) 21, was associated with worsening cognitive decline in DLB [67]. In support of this, evaluation of neuronal loss and neuropathology in DLB has shown that there is minimal neuronal loss in the superior temporal sulcus in DLB without concomitant AD pathology [70]. A prospectively studied autopsy cohort of patients with DLB showed that the severity of AD pathology was detrimental to prognosis, although within the context of an additive effect with α-syn [71]. The neuropathology of AD will be explored further in section 1.2.4.

In addition to the types of pathology stated above, a further type of α-syn pathology has been revealed in recent years. Rather than localising to LB, pre-synaptic aggregates of α-syn have been identified in both DLB and PD. In fact, it has been proposed that over 90% of α-syn pathology is located in the pre-synapse rather than in LB [72]. This theory raises the possibility that synaptic dysfunction caused by pre-synaptic α-syn aggregates, rather than LB, may be the offending agent when it comes to neurodegeneration and symptomatology in DLB [72-74]. This is supported by evidence that LB density fails to correlate with disease duration, cognitive decline or severity of symptoms in DLB [75, 76]. However, it remains to be confirmed whether associations exist between pre-synaptic aggregates of α-syn and clinical phenotype in DLB.

Macroscopically, DLB is characterised by relative preservation of cerebral grey matter, as measured by structural brain imaging [77]. In a notable prospective imaging study which benefited from pathological confirmation of diagnosis, Burton et al. reported a strong correlation between hippocampal atrophy and Braak ptau stage, but not LB, suggesting that cortical volume loss in DLB could be caused by concomitant AD pathology [78]. In support of this, another imaging study revealed that DLB was characterised by a lack of global cerebral atrophy, with severity of atrophy found to be similar to controls. The same study found that, in contrast, AD cases and mixed DLB/AD cases had

significantly higher rates of global cerebral atrophy. This suggests that DLB may not be characterised by significant cerebral atrophy without the presence of co-existing AD pathology [79].

### 1.1.6 Management

The primary focus of medical treatment of DLB is symptomatic and based on limited clinical trial data, with the majority of evidence being inferred from trials in AD and PD [80].

The latest consensus statement published by the British Association for Psychopharmacology (BAP) concisely presents current evidence for the medical management of DLB [81]. There is now a good body of evidence to support the use of cholinesterase inhibitors in the treatment of DLB. Original clinical trials included in the BAP guidelines have shown benefits in taking cholinesterase inhibitors for a variety of neuropsychiatric symptoms in DLB, including cognition, attention and visual hallucinations [82, 83], as well as reducing carer stress [84]. Donepezil, a cholinesterase inhibitor, has now been licenced for use as a treatment for DLB in Japan and the Philippines, but similar approval has not yet been granted in the UK or United States of America. However, another cholinesterase inhibitor named Rivastigmine has been licensed for use in PDD in the UK [81].

DLB also presents with non-psychiatric symptoms that are important to recognise and for which treatments are available. A recent review article states that effective treatments are available for many non-cognitive features of DLB, including for Parkinsonism, autonomic instability and REM behaviour sleep disorder [85]. Specifically, Levodopa can help with Parkinsonism in DLB [86] and Clonazepam can be effective for REM sleep behaviour disorder [87].

Particular caution should be used when treating DLB patients with neuroleptic medication. Antipsychotic medications, particularly the older typical antipsychotics, have been shown to cause severe Parkinsonism, coma and increased mortality in DLB [88]. Their use in DLB should be avoided where possible and only utilised when all other strategies to manage behavioural symptoms have been exhausted [88, 89].

Non-pharmacological options are also an important aspect of the management of DLB, however there is limited evidence in this area due to a paucity of randomised controlled trials that have included patients with DLB. Techniques that could be employed include: modifying the home environment, reflecting on how caregivers respond to challenging behaviours, ensuring symptoms are distressing before rushing into medicating, educating caregivers and ensuring utilisation of available support services [90]. A review article in this area suggests that the following interventions could be helpful in LBDs: psychological interventions to reduce distress caused by visual hallucinations, physical exercise to improve gait, and music therapy to reduce distress [91]. Despite the lack of confirmatory evidence, all of the interventions listed above appear to have face validity in the non-pharmacological management of DLB and most are unlikely to cause serious harm. In addition to the interventions described above, general advice for people living with dementia should include guidance on proper financial planning, social care planning and managing potential risks including gas safety, wandering and driving.

Lastly, several novel disease-modifying treatments are being considered for the spectrum of LBDs. Immunotherapy against α-syn is being trialled for the treatment of PD [92], with results awaited from phase 2 clinical trials. In addition, deep brain stimulation has been approved in the USA for treatment of PD and is currently being trialled as a treatment for DLB [93].

### 1.1.7 Prognosis

DLB has been shown to have a poorer prognosis compared with AD, with higher healthcare costs, greater caregiver stress, poorer quality of life and decreased time to death [94]. In addition, patients with DLB have been shown to be admitted to general hospitals more frequently compared with those with AD and the general elderly population, with infections and falls being the main discharge diagnoses [94, 95].

A large multinational study examining the rate of cognitive decline in DLB showed that patients with DLB declined at a rate of 2.1 Mini-Mental State Examination (MMSE) score points per year. This was compared to 1.6 points per year in AD and 1.8 points per year in PDD [96]. Additionally, time to reach severe dementia has been shown to be significantly shorter in DLB compared

with AD [97]. In fact, the rate of cognitive decline may be worst in those with mixed AD and LRP, as shown by a study of post-mortem confirmed mixed cases examined with a retrospective review of clinical notes [98].

## 1.2 Alzheimer's disease

Alzheimer's disease is named after the German psychiatrist Alois Alzheimer, who in 1907 published a paper reporting the findings of a post-mortem examination on Auguste Deter, a 55 year old woman who had died from progressive behavioural and memory disturbance [99]. Alzheimer noted the presence of two distinctive pathologies in Deter's brain, which will be described in the neuropathology section below (1.2.4).

### 1.2.1 Epidemiology

AD is the most common cause of dementia, accounting for approximately two-thirds of all cases [100]. The prevalence of AD in the UK has been estimated to be 500,000 people [3]. However, it has been proposed that the UK incidence of new cases of dementia, including AD, may in fact be decreasing, possibly as a result of improved management of vascular risk factors and higher levels of educational attainment [101].

### 1.2.2 Aetiology

The biggest risk factor for developing AD is increasing age, with incidence and prevalence rates increasing exponentially with age [100]. Several other medical, lifestyle and genetic factors have also been implicated in the aetiology of AD.

Autosomal dominant familial AD accounts for approximately 1% of all cases of AD, with the age of dementia onset typically younger than 65 years old [102]. Mutations in three genes (Amyloid Precursor Protein - *APP*, Presenilin 1 – *PSEN1* and Presenilin 2 – *PSEN2*) have been identified as having high penetrance for early onset AD [103]. These genetic mutations all have the same downstream

effect, which is to increase production of Aβ that aggregates to form plaques in the diseased brain.

Twin studies have revealed that the genetic heritability of late onset AD (defined as onset later than 65 years old) is approximately 60% [104]. The strongest known genetic risk factor relates to the *APOE* gene. One copy of the *APOE* ε4 allele is associated with a 3-fold increased risk of developing late-onset AD, with two copies increasing risk by 12-fold [105]. Although possession of the ε4 allele is neither mandatory nor sufficient for the development of AD, it has been shown that carrier status is also associated with an earlier onset of the disease [106].

In recent years, GWAS have shown that in addition to *APOE*, a number of common genetic polymorphisms with small effect sizes, related to inflammatory processes and cholesterol metabolism, are risk factors for late-onset AD [107-109]. Furthermore, polymorphisms in "triggering receptor expressed on myeloid cells 2" (*TREM2*) has been shown to have a large effect size for risk of AD, but is relatively rare in the population [110, 111].

Other known risk factors for AD include traumatic head injury [112], mid-life hypertension, type 2 diabetes mellitus, lower educational level and obesity [100, 113]; some of which are associated with vascular disease and chronic inflammation. These epidemiological studies and GWAS suggest a key role for inflammation in the aetiology and pathogenesis of AD, a topic which will be explored further in section 1.5.

### 1.2.3 Clinical features and diagnosis

AD typically presents clinically with an insidious onset of dementia and gradual progression. Amnesia is usually the most prominent early feature of AD but other cognitive domains can also be affected, including language, visuospatial ability and executive function. Neuropsychiatric symptoms, including agitation and apathy, are also common features of AD, particularly as the disease progresses. As well as gathering history from the patient and reliable informant, clinicians also often use cognitive assessment tools to objectively quantify cognitive performance. There are several tools available, including the MMSE [114] and Montreal Cognitive Assessment (MoCA) [115].

In recent years there has been increasing focus on prodromal dementia states, including prodromal AD. Mild cognitive impairment (MCI) can be defined as cognitive dysfunction that is greater than expected for an individual's age but that does not interfere with activities of daily living [116]. Patients with MCI have an increased risk of conversion to dementia, although the precise conversion rate is subject to debate. One study showed that approximately 50% of people with MCI progressed to dementia within 5 years, but some people appeared to remain stable or even return to normal over time [116]. Another study showed the conversion rate of MCI to dementia to be 45% within 7 years [117], with yet another demonstrating an annual conversion rate of 13% for a sample of memory clinic patients [118]. People with the amnestic subtype of MCI, who present with prominent deficits in episodic memory, have a particularly high risk of progression to AD and therefore amnestic MCI is considered to be a prodromal stage of AD [116, 119].

The National Institute of Aging – Alzheimer's Association (NIA-AA) clinical diagnostic guidelines for probable AD are widely cited and used [6], and are summarised in table 1.2.

**Table 1.2: NIA-AA clinical diagnostic criteria for AD**

| | |
|---|---|
| **All-cause dementia** | Dementia is diagnosed when there are cognitive or behavioural symptoms that:<br>• Interfere with the ability to function at work or at usual activities<br>• Represent a decline from previous levels of functioning and performing<br>• Are not explained by delirium or major psychiatric disorder;<br>• Cognitive impairment is detected and diagnosed through a combination of (1) history taking from the patient and informant and (2) an objective cognitive assessment<br>• The cognitive or behavioural impairment involves a minimum of two cognitive domains (memory, reasoning/judgement, visuospatial ability, language, personality/behaviour) |
| **Probable AD dementia** | • Insidious onset<br>• Clear-cut history of worsening cognition by report or observation<br>• The initial and most prominent cognitive deficits are evident on history and examination in one of the following categories: amnesia, language, visuospatial, executive dysfunction<br>• The diagnosis of probable AD should not be applied if there is evidence of substantial concomitant cerebrovascular disease, core features of DLB, prominent features of FTD, prominent features of primary progressive aphasia or evidence for any other concurrent neurological disease that could have a substantial effect on cognition |

Adapted from McKhann et al. 2011 [6]

Imaging biomarkers are now widely used to support the diagnosis of AD. Cerebral atrophy on structural brain imaging, with a predominance for hippocampal volume loss, has been shown to be a biomarker for AD [120]. Impaired glucose metabolism and impaired perfusion in the temporal and parietal lobes have also been shown to be biomarkers for AD using functional brain scans such as PET or SPECT [121]. Amyloid PET imaging is now commonly used in clinical trial settings to confirm the presence of cerebral Aβ

in patients with probable Alzheimer's disease [122]. In addition, decreased Aβ and an increase in the ratio of ptau to total tau in cerebrospinal fluid (CSF) can support a diagnosis of AD, but these CSF biomarkers have limited value when attempting to differentiate AD from other causes of dementia [123].

It should be noted that the diagnosis of AD, and indeed other causes of dementia, can only be verified at post-mortem upon neuropathological examination of the brain.

### 1.2.4 Neuropathology

Macroscopically, the most striking neuropathological feature of AD is cortical atrophy, particularly of the medial temporal lobes [124]. Interestingly the extent of cortical atrophy, along with the rate of worsening atrophy over time, has been shown to be greater in AD than in DLB [77].

It is, however, only when looking under the microscope that the cardinal features of AD are found, namely Aβ plaques and ptau NFTs [125]. These two characteristic findings are shown in figure 1.3 and described in more detail below.

Aβ is a 40-42 amino acid peptide that is derived from the transmembrane amyloid precursor protein (APP), after it is sequentially cleaved by β-secretase and γ-secretase [126], and which can aggregate in the form of plaques. In AD, Aβ accumulates to form synaptotoxic soluble oligomers and also insoluble amyloid fibrils that make up extracellular plaques. These Aβ plaques can be classified as either neuritic or diffuse [124]. Neuritic plaques consist of a dense amyloid core and are surrounded by dystrophic neurites (dendritic accumulation of ptau), microglia and astrocytes, whereas diffuse plaques are characterised by a lack of neurites and glia. Other features of AD pathology include neuropil threads (consisting of ptau) and cerebral amyloid angiopathy (Aβ accumulation in the lumen wall of cerebral vasculature) [124, 125].

NFTs are intracellular aggregations of ptau, a microtubule-associated protein. A review of clinico-pathological studies has shown that the presence and load of NFTs correlates positively with cognitive decline in AD, and in fact does so better than Aβ plaque burden [127].

The NIA-AA guidelines for the neuropathological assessment of AD recommends that specific brain regions be evaluated for AD pathology, and also describes preferred methods for immunohistochemistry. The guidelines recommend that the severity of AD neuropathology be measured using three parameters: amyloid load, Braak NFT stage and CERAD (neuritic plaque score) to obtain an "ABC" score [125].

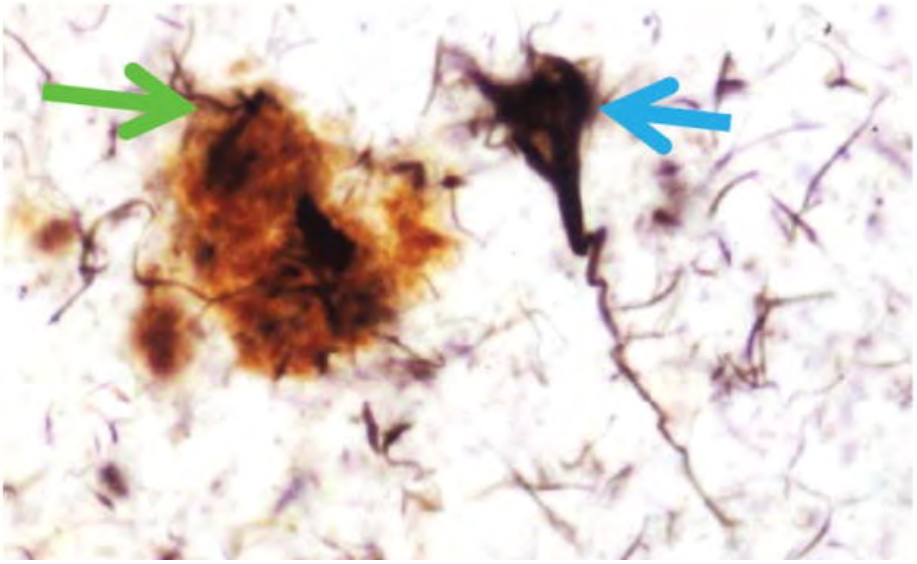

**Figure 1.3: AD pathology**
Digital image taken of the human cortex of a patient with AD. Double immunohistochemistry labelled for Aβ (brown) and ptau (black) showing evidence of an Aβ plaque (green arrow) and neurofibrillary tangle (blue arrow) respectively. Neuropil threads can also be seen, most prominently associated with the neurofibrillary tangle. Image taken from Nelson et al. 2012 [127].

### 1.2.5 Management

Currently there are three cholinesterase inhibitors (Donepezil, Galantamine and Rivastigmine) and an N-methyl-D-aspartate (NMDA) receptor antagonist (Memantine) licenced in the UK for use in the treatment of AD. Donepezil, Rivastigmine and Galantamine are licensed for mild to moderate AD, with Memantine licenced for moderate to severe AD. A meta-analysis has confirmed that they are effective in improving function and behavioural symptoms in AD [128], with all four medications included in the latest consensus statement published by the BAP [81]. Antidepressant medication is frequently used for the management of depression in AD, despite limited evidence [129]. Anti-

psychotic medication can be used for treatment of behavioural symptoms in AD, but a meta-analysis of randomised controlled trials has shown that they cause increased mortality when used in people with dementia [130].

There are currently dozens of clinical trials underway, from phase I to phase III, testing the use of various agents in the treatment of AD, with many drugs believed to be disease-modifying [131]. There was initially great hope that immunotherapies targeting the removal of Aβ or ptau could halt or even reverse dementia in AD. This mainly stemmed from the amyloid cascade hypothesis [132], which postulates that Aβ aggregates are the causative agent of AD and that all other features are downstream, including the formation of NFT, neurochemical alterations, inflammation, synaptic dysfunction, neuronal cell death and ultimately dementia [133]. However, several randomised clinical trials have tested this theory by targeting components of the amyloid pathway and have failed to meet their primary end-points, prompting some to question this hypothesis [134]. There have also been significant safety concerns about some of the immunotherapies, including increased frequencies of amyloid-related imaging abnormalities (ARIA) in treatment subjects [135].

As with all other causes of dementia, non-pharmacological options are an important aspect of the management of AD. Treatments with modest evidence include cognitive training, cognitive rehabilitation and cognitive stimulation therapy [136]. As in DLB, carer support and education, along with thorough management of risks and advice on planning for the future, are all also worthwhile interventions in AD.

## 1.3 Overlap between DLB, AD and PDD

Despite the specificity for the clinical diagnosis of DLB being as high as 79-100% [137], several studies have suggested that despite widespread adoption of the 2005 consensus criteria for diagnosing DLB, sensitivity may be as low as 32-88% [138-140]. This is likely to be due to the overlap of clinical symptoms between DLB, AD and PDD leading to challenges in differentiating these diseases in clinic. The latest consensus criteria for diagnosing DLB were only

published in late 2017 and it remains to be established whether they further improve the sensitivity and specificity of the clinical diagnosis of DLB [18].

Differentiation of DLB from PDD in clinical terms is defined by the timing of onset of motor features of PD relative to the onset of dementia. DLB is diagnosed if a dementia syndrome develops prior to, or within 1 year after, the onset of Parkinsonism. Conversely, PDD is diagnosed if a dementia syndrome develops at least one year after the onset of Parkinsonism [9].

As well as overlap in clinical features, there is also significant overlap of neuropathology between AD, DLB and PDD. It has been shown that Aβ plaques, and to a lesser extent ptau NFTs, are commonly found in DLB as revealed in neuropathology studies [67, 69] and amyloid PET imaging studies [141], but typically at lower levels than found in AD [142, 143]. The Walker et al. study [69] also showed significant presence of LRP in cases clinically diagnosed as AD, further demonstrating overlap of pathology between AD and DLB. Moreover, a large population-based neuropathological study revealed that a significant proportion of elderly non-demented controls showed evidence of LRP [144]. There is also evidence of a strong overlap of neuropathology in PDD, with evidence of significant α-syn, ptau and amyloid pathologies present [145]. A recent paper by Howlett et al. [67] elegantly illustrates the overlap in neuropathology in AD, DLB and PDD, as shown in figure 1.4.

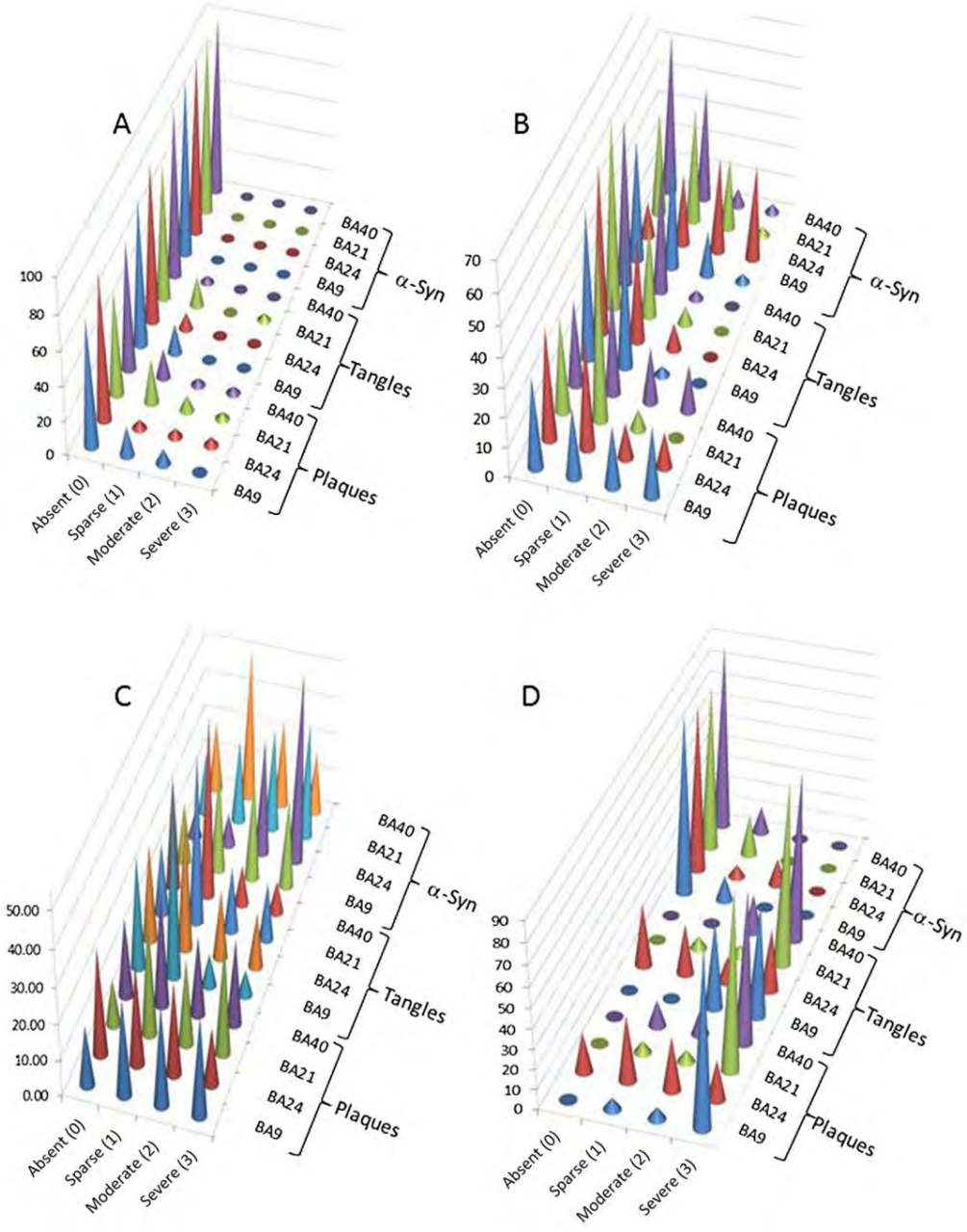


**Figure 1.4: Frequency of pathology scores in controls, PDD, DLB and AD**
Frequency of pathology scores in (A) controls, (B) PDD, (C) DLB and (D) AD. Y-axis shows percentage of cases with a particular score, X-axis shows the severity of pathology and Z-axis shows the area of brain sampled along with the type of neuropathology found. Image taken from Howlett et al. 2015 [67].

Cerebrospinal fluid (CSF) studies have shown that up to a quarter of DLB patients have a CSF profile consistent with AD [146]. Interestingly, those DLB patients who have CSF evidence of concurrent AD pathology appear to show a faster decline in cognitive function [147] and a higher risk of mortality and institutionalisation [148].

There is also evidence of neurochemical overlap between these diseases. There is a significant cholinergic deficit in AD, but it has been shown that this is even more prominent in DLB [41-43], supporting the use of cholinesterase inhibitors in both diseases. Impairment of cortical serotonin levels has also been found in both DLB and AD [149], with novel medications being trialled targeting the serotoninergic system in both diseases. However, recent reports have shown that these trials have failed to show significant benefits in either AD [150] or DLB [151].

The overlap in clinical features, neuropathology and neurochemistry, along with the shared risk genes between DLB, AD and PD, suggests that these diseases may share some underlying pathophysiological mechanisms. However, it should also be considered as to whether any overlap detected in genetic or clinical studies could result from possible diagnostic misclassification.

Ensuring that the correct dementia diagnosis is made is important for a number of reasons. Patients with DLB are particularly sensitive to antipsychotic medication, leading to increased mortality [152]. In addition, patients with DLB have a more impaired quality of life and require significantly more resources to manage compared to AD patients. Indeed, one in four caregivers rank having DLB as worse than death [153, 154] and caregivers also report particularly high levels of distress [155, 156]. Furthermore, DLB causes significantly greater functional disability than AD, with this finding likely to be related to their greater extrapyramidal motor dysfunction [157].

Gaining a better understanding of the aetiology of DLB may lead to improved accuracy of diagnosis leading to more tailored care for patients, whilst also opening avenues for the development of novel treatments for the disease. In addition, this will allow the development of more accurate and responsive models of medical and social interventions for dementia care.

## 1.4 The immune system

The human body is subject to a variety of insults from the environment, some of which are pathogenic. Epithelial structures such as the skin provide a physical barrier to prevent direct entry of these pathogens. However, if this barrier is breached then the immune system is activated in order to defend the body and also co-ordinate repair following injury. It does this by being able to differentiate molecules and cells as either "self" or "non-self". The immune system can be divided functionally into two parts: innate immunity and adaptive immunity, although there is significant cross-talk between them.

### 1.4.1 Innate immunity

Innate immunity is generally non-specific and rapid in responding to threats, and is usually activated immediately upon infection or injury, resulting in "inflammation". Cells and molecules of the innate immune system include phagocytic cells (e.g. macrophages), natural killer cells and complement. Complement is a cascade of molecules that target foreign agents and can trigger cell death through lysis. Innate immune cells are able to rapidly recognise stimuli using pattern recognition receptors (PRRs) to identify pathogenic molecules known as pathogen associated molecular patterns (PAMPs) and molecules indicating damaged tissue known as damage associated molecular patterns (DAMPs). This enables the pathogen to be destroyed though a process called phagocytosis, or alternatively to allow co-ordination of the removal and repair of damaged tissue [158].

Microglia are the innate immune cells of the brain and are discussed further in section 1.4.3.

### 1.4.2 Adaptive immunity

In contrast to innate immunity, adaptive immunity takes longer to be activated but is more specific in its response. Components of the adaptive immune system include dendritic cells, lymphocytes and antibodies. Dendritic cells function to process antigen material before presenting it on their cell surface for detection by lymphocytes, thus acting as messengers between the innate and adaptive immune systems. This "antigen-presenting" function of dendritic

cells is shared by macrophages, and leads to activation and differentiation of lymphocytes. Lymphocytes can be differentiated into B lymphocytes (or B cells) and T lymphocytes (or T cells).

There is extensive interplay between different subsets of the adaptive immune system. B cells bind to antigen and can differentiate into effector plasma cells that produce antibody. Antibodies perform their immune function in two main ways. Firstly, they can bind directly to antigen associated with pathogens and prevent passage of pathogens into host cells, in a process called neutralisation. Secondly, antibodies can coat the surface of a pathogen to promote phagocytosis, with this process named opsonisation. The process of antibody-mediated immunity is also called humoral immunity [158].

Receptors on the T cell surface can also bind with antigen and trigger differentiation of T cells into cytotoxic T cells (that kill cells infected with intracellular pathogens) or helper T cells (which help to co-ordinate the immune response). Cytotoxic T cells can be identified by the presence of a CD8 receptor on their cell surface, with CD4 receptors identifying helper T cells. It is notable that helper T cells can also trigger activation of antigen-stimulated B cells to influence their production of antibody. In addition, activated B cells and T cells can differentiate into memory cells to allow long-lasting immunity following subsequent exposure to the same antigen. Indeed, one of the key features of adaptive immunity is memory of previous pathogenic infections to allow for a more rapid and stronger response to a subsequent infection [158].

The adaptive system utilises a large group of chemical messengers in order to allow communication between lymphocytes and also with innate immune cells. These chemical messengers are called cytokines, and include the interleukins (IL1-IL24), tumour necrosis factors (TNFs) and transforming growth factors (e.g. TGF$\beta$) [159]. The actions of cytokines are often complex but overall their function is to mediate cellular communication. IL1, IL6 and TNF$\alpha$ are generally considered to be pro-inflammatory, while IL4, IL10 and IL13 are known to modulate anti-inflammatory functions [159]. The alteration of the profile of lymphocytes and cytokines has been implicated in ageing and several neurodegenerative disorders.

### 1.4.3 Cerebral inflammation

Neuroinflammation refers to the activation of immune cells in the central nervous system (CNS) as a result of injury, infection or tissue damage. Microglia are the resident macrophages of the CNS and play a fundamental role in cerebral innate immunity, as well as initiating recruitment of peripheral adaptive immune cells through antigen presentation and release of cytokines. Microglial cells are of myeloid lineage and during neurodevelopment they migrate into all areas of the CNS [160]. The total microglial population is thought to remain stable over the course of a lifetime, but with significant self-renewal and turn-over [161]. *In-vivo* time-lapse imaging studies have revealed that microglial processes are remarkably motile, continuously undergoing rapid cycles of formation out from, and withdrawal back to, the cell body. [162]. Overall, microglia are thought to be highly mobile and reactive, even in the absence of neurological disease, as they survey their microenvironment for pathogens and injury [160, 162, 163].

Microglia, like all macrophage cells, have been historically classified into two major activation states: 1) Classical activation (termed M1) represents a pro-inflammatory phenotype, and 2) Alternative activation (termed M2) representing either a regulatory, anti-inflammatory or healing phenotype. However, this dogma has been challenged in recent years and it is now widely accepted that macrophages display incredible plasticity and are able to change their phenotype in response to environmental stimuli [164]. The spectrum of potential macrophage activation states is illustrated in figure 1.5. Indeed, it is clear that microglia rapidly adapt their phenotype in response to disturbance in their microenvironment and display a range of functional states [163, 165, 166]. Some of the known functions of microglia are presented in a list below, adapted from a review by Boche et al. [166].

- Phagocytic activity during neuronal/synaptic development
- Recognition of cell surface antigens on pathogens, known as PAMPs, such as the endotoxin lipopolysaccharide (LPS)
- Ingestion and lysosomal destruction of: damaged cells, neurons, micro-organisms and virally-infected cells

- Presentation of antigen bound to Major Histocompatibility Complex class II (MHC) for activation of T lymphocytes
- Response against antibody-antigen complexes
- Modulation of the immune response
- Removal of cell debris to facilitate plasticity and synaptogenesis

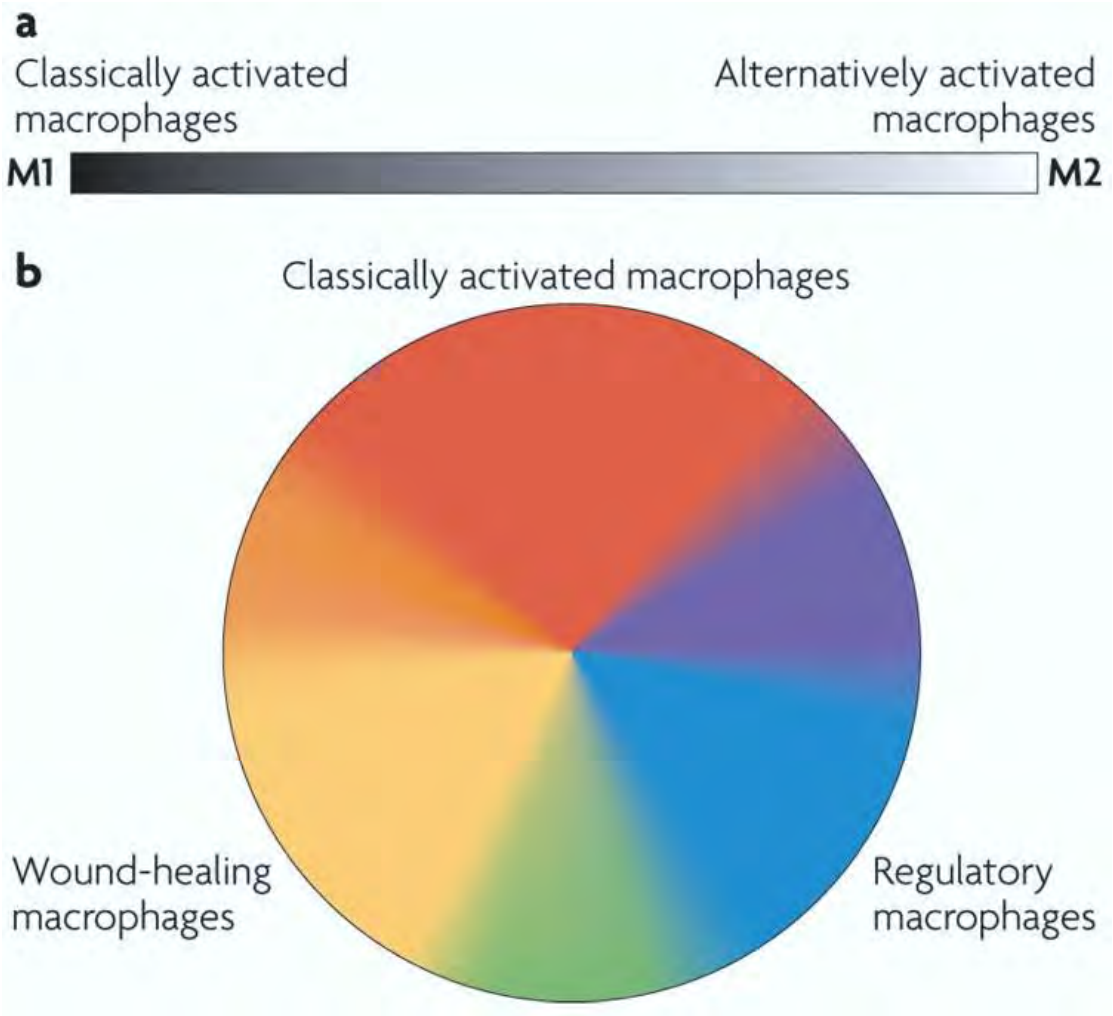


**Figure 1.5: Illustration of the spectrum of macrophage activation**
A: Depiction of the historical nomenclature used to describe two macrophage activation states on a linear scale. B: Depiction of three macrophage activation states, with significant overlap. Image taken from Mosser et al. [164].

Activation of microglia can be assessed by observing a number of different variables. Firstly, altered cell morphology can provide evidence of the type of microglial phenotype, with ramified microglia (defined by short, fine processes) thought to indicate a surveillance phenotype. In contrast, amoeboid-shaped

microglia are thought to indicate a phagocytic phenotype [166]. Furthermore, microglia with "beading" of their processes have been proposed as being dystrophic, possibly as a result of ageing [167]. However, assessment of microglial phenotype by observing morphology alone may be limited by issues such as assessor objectivity and training. Furthermore, microglia have been shown to rapidly switch their phenotype *in vivo* without obvious changes in their morphology [168].

In addition to morphological changes, activation of microglia can be assessed using immunohistochemistry against a range of defined markers. A number of markers have been previously used to investigate the immunophenotype of microglia [166], with ionized Calcium-binding adapter molecule 1 (Iba1), human leukocyte antigen – antigen D related (HLA-DR) and cluster of differentiation 68 (CD68) the most extensively used microglial markers researched in AD [169]. Table 1.3 shows a summary of markers commonly used to assess microglial immunophenotype.

**Table 1.3: Markers used to assess immunophenotype of microglia**

| Marker | Detection | Function |
|---|---|---|
| **Iba1** | Ionised calcium-binding adaptor molecule 1 on resting and activated microglia [166, 170, 171] | Motility, upregulated in inflammation |
| **HLA-DR** | Cell surface homologue of MHCII [166, 172] | Antigen presentation, upregulated in inflammation |
| **CD68** | Microglial lysosomes [166] | Phagocytic activity |
| **CD64** | High affinity receptor for immunoglobulin [173] | Co-ordination of phagocytosis and inflammatory response |
| **CD32** | Low affinity receptor for immunoglobulin [173] | Co-ordination of phagocytosis and inflammatory response |
| **CD16** | Low affinity receptor for immunoglobulin [173] | Lysis of pathogens |

Adapted from Boche et al. 2013 and Minett et al. 2016 [166, 174]

One method by which microglia can become activated is through engagement of cell surface receptors named Fc gamma receptors (FcγR). This family of receptors bind to the constant domain of antibody, also known as immunoglobulin (Ig), and mediate the effector cell response upon detection of immune complexes [175]. They are present on cells throughout the immune system, including microglia [173], and also on perivascular macrophages and neurons in the CNS [175]. Human FcγR can be subdivided into the activating FcγRI (alternatively named CD64), FcγRIIa (CD32a) and FcγRIIIa (CD16a), the inhibitory FcγRIIb (CD32b) or the decoy receptor FcγRIIIb (CD16b). FcγRI (CD64) in particular has the highest affinity to the Ig subclass IgG [176] and is the only FcγR that can bind to monomeric IgG [177].

The precise level of activation of immune cells via FcγR depends on the ratio of activating versus inhibitory receptors on the surface of each cell, allowing for a tightly regulated immune response. Antibody binding to FcγR on innate immune cells leads a pro-inflammatory response and phagocytic phenotype, which can also lead to destruction of surrounding healthy tissue [173]. Furthermore, when this equilibrium is disturbed in cells that have a higher ratio of activating to inhibitory FcγR, these cells are even more likely to produce an uncontrolled immune response, causing additional tissue damage [175].

The notion that microglial activation is always harmful is almost certainly too simplistic. It is likely that there are both helpful and destructive effects of microglial activation, with a continuous balance between pro-inflammatory and anti-inflammatory phenotypes, depending on various factors. It may be that the balance tips towards a harmful phenotype with age and in neurodegenerative diseases, and with increasing neuropathology. It has also been proposed that microglia may become dysfunctional with age, causing diminished neuroprotective functions and downregulated phagocytic activity. Along with an increase in secretion of inflammatory cytokines, this could lead to neurodegeneration through enhanced neuronal loss and protein aggregation [171, 178]. However, the exact mechanisms between which neuroinflammation and neurodegeneration interact have yet to be categorically established.

### 1.4.4 Communication between peripheral and cerebral inflammation

Despite the brain once being thought of as an immune-privileged organ, it is now known that peripheral inflammation communicates extensively with the brain. One such example of communication is the presence of symptoms of "sickness behaviour" during periods of systemic infection, triggered by peripheral cytokines [179]. Such behavioural symptoms include increased anxiety, depression and apathy, and have been shown to be associated with increased serum pro-inflammatory cytokines in AD [180]. However, most inflammatory mediators are too large to pass through the blood-brain barrier (BBB) directly into the brain parenchyma [181].

The precise mechanism of communication between peripheral and cerebral immunity remains subject to debate, but there are several possible routes of communication to consider. Firstly, peripheral inflammatory events can be signalled to the brain via the sensory fibres of the vagus nerve. Secondly, blood cytokines can communicate directly with cells of circumventricular organs of the brain, which lack an intact BBB. Lastly, cytokines may communicate through the BBB via endothelial cells or perivascular macrophages [168, 182] in cerebral vasculature. In addition, there is evidence that a small proportion of peripheral IgG can cross the BBB [183, 184], implying that a peripheral humoral immune response could trigger the activation of microglia.

In recent years it has been hypothesised that microglia can be primed, resulting in an exaggerated and harmful response to secondary peripheral stimuli. In aged mice, microglia show enhanced sensitivity to stimuli, demonstrated by upregulation of cell surface receptors and an exaggerated response to secondary inflammatory stimuli, typically from outside the brain [185, 186]. Once the secondary stimulus has been communicated to the CNS, primed microglia are thought to respond more robustly and show exaggerated production of pro-inflammatory cytokines, contributing to increased phagocytosis and neuronal damage [187-189]. This theory has been illustrated by Perry and Teeling in figure 1.6.

The cause of microglial priming may be due to a number of factors, including age-related changes to the cerebral microenvironment, the presence of neuropathology in the aged brain, or the presence of chronic low-grade

systemic inflammation [185, 189]. These possible causes are particularly relevant to an elderly population, who are likely to have increased neuropathology and are likely to have been subjected to repeated infection or trauma, along with possessing a high prevalence of co-morbidity including chronic inflammatory conditions that are associated with chronic low-grade systemic inflammation.

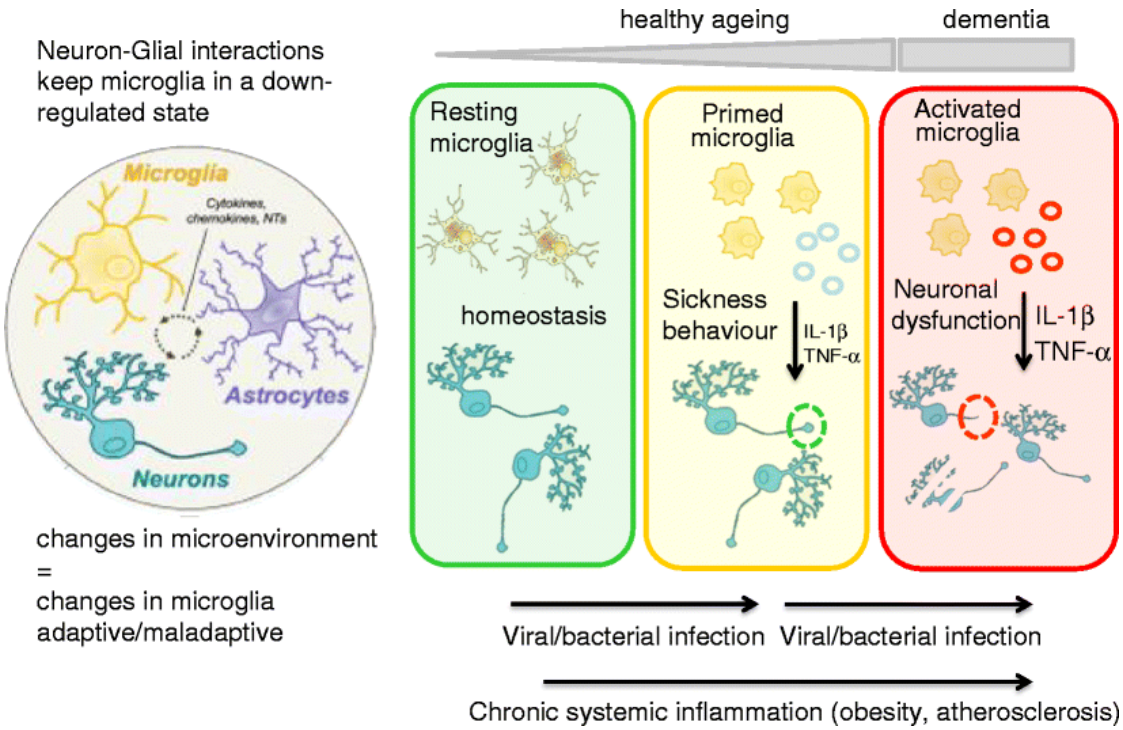


**Figure 1.6: Illustration of microglial priming in aging and dementia**
Image taken from Perry and Teeling 2013 [189]

## 1.5 Inflammation in AD

The concept of increasing impairment of the immune system with age has been termed "inflammaging" [190]. It is thought to result in chronic, low-grade activation of the innate immune system, leading to inflammatory cytokine production [191, 192], and may develop as a consequence of cumulative exposure to antigen across many decades [191]. Indeed, the expression of inflammation-related genes has been shown to be robustly upregulated with

ageing in cognitively normal individuals, especially for genes involved in innate immunity [193].

The most marked changes that occur with age appear to be related to the adaptive immune system, in a process termed immunosenescence, which literally means the deterioration of immunity with age [194]. Age-related changes to the adaptive immune system include a shift in the proportion of T cells from naïve to memory. In addition, chronically-activated T cells can become "worn-out" and functionally dormant [194, 195], while B cell populations are also diminished [196]. Overall, aging can lead to chronic, low-grade activation of the innate immune system and an impaired adaptive immune response.

Aging has been shown to be related to an increase in glial activation, inflammatory markers and brain atrophy [197]. The process of aging itself is therefore associated with increased inflammation. Age is known to be the biggest risk factor for developing sporadic AD, and so there has been an increasing focus on the role of inflammation in this disease. There is now a significant body of evidence supporting a key role for inflammation in the aetiology and progression of AD, as will be reviewed in the following sections.

### 1.5.1 Pre-clinical evidence

Cell culture and animal work has supported the hypothesis that inflammation plays a significant role in the aetiology of AD. Microglia have been shown to surround, react to, and phagocytose Aβ in cell cultures and in rodent models of AD [198]. However, in transgenic mice it has also been shown that despite microglia surrounding Aβ and actively phagocytosing Aβ, they ultimately fail to completely clear those plaques [199]. Furthermore, a study in mice with chronic neurodegeneration showed that activated FcγR expression was increased following initiation of peripheral inflammation by a bacterial mimic, and that there was an associated increase in cerebral Ig following the peripheral infection [200]. This showed that systemic inflammation, in the context of chronic neurodegeneration, could increase cerebral Ig concentrations and expression of FcγR on microglia, possibly resulting in increased antibody-mediated phagocytosis by microglia.

Murine models of AD have shown that non-steroidal anti-inflammatory drugs reduce the level of Aβ plaque load [201], providing evidence that modifying peripheral inflammation in mice reduces AD pathology. However, there are clearly countless differences between the immunological profile of human AD and rodent models of the disease, which mean that caution should be applied when interpreting data from studies examining inflammation in rodents [202].

### 1.5.2 Genetic evidence

In recent years, several large scale GWAS in AD have received much attention from the academic field. They have shown that a number of genetic polymorphisms involved in cholesterol metabolism and inflammatory processes are risk factors for the development of sporadic AD [107-109].

One such polymorphism is of the *CD33* gene, a variant of which increases the risk of developing AD [203]. Whilst the precise function of CD33 is not clear, it has been shown to be expressed by microglia, and the number of CD33 positive microglia is higher in AD brains, with a positive correlation with Aβ plaque burden [204]. This suggests that CD33 plays a role in the accumulation of, or failure to clear, Aβ plaques in the AD brain.

A notable polymorphism that has been shown to give a three- to five-fold increase in the risk of AD is in *TREM2*, although the alleles identified are much rarer than *APOE* ε4 in the general population [110, 111]. TREM2 is known to be highly expressed on microglial cells in animal models of AD. The physiological role of TREM2 is still not well defined, but it may play a role in regulating inflammatory processes in the brain with a particularly important function in the clearance of apoptotic neurons [205].

Other genetic studies have identified polymorphisms in several genes associated with inflammation that increase risk of late-onset AD, such as *IL1β* [206], *HLA-DR* [207], *CLU* [108] and *CR1* [109]. *CLU* codes for a protein called clusterin (also known as *APOJ*), while the gene *CR1* codes for “complement receptor 1”. Both *CLU* and *CR1* have been shown to be involved in the innate immune response [108].

The identification of numerous inflammation-related genes, as reviewed above, only strengthens the theory that inflammation occurs not just as a consequence of neurodegeneration, but that it plays a fundamental role in the aetiology of dementias such as AD.

### 1.5.3 Epidemiological evidence

Epidemiological studies have shown that chronic inflammatory conditions, such as mid-life atherosclerosis, obesity and type 2 diabetes mellitus, are risk factors for the development of AD [208-212]. While each of these risk factors may have a small effect, their combined cumulative effect over time may be more significant [213]. This indicates that the number of chronic inflammatory conditions may incrementally increase risk of developing late-onset AD. Supporting this, several epidemiological studies have shown reduced risk of developing AD in people taking NSAIDs, as reviewed in section 1.5.7.

Another condition of interest in this field is delirium. Systemic infections are a major cause of delirium in the elderly, and a significant association has been found between AD and delirium. The incidence of developing dementia two years after a diagnosed episode of delirium in healthy aged controls has been shown to be as high as 55% [214]. In addition, the presence of one or more infections over the course of 5 years in a large General Practice (GP) database showed a two-fold increase in the odds of developing AD [215].

Overall, it appears that there is significant population-based evidence that peripheral inflammation is an important risk factor for AD. This again supports the hypothesis that alterations in peripheral inflammation precedes onset of neurodegenerative diseases such as AD.

### 1.5.4 Imaging evidence

The development of imaging ligands has allowed the possibility of examining cerebral inflammation *in-vivo*. The PK11195 ligand measures upregulation of TSPO (translocator protein), which has been identified as a marker of microglial activation that can be imaged using PET. PK11195 signal has been shown to be significantly higher in cortical regions in patients with AD compared with controls [216, 217]. Interestingly, a study by Edison et al. showed that MMSE

score correlated negatively with PK11195 signal but was not associated with Aβ PIB-PET ligand signal [217]. This raises the possibility that microglial activation may in fact be a more prominent factor in neuronal degeneration than the presence of Aβ.

Evidence of microglial activation using PK11195 has not only been demonstrated in AD, but in a number of other neurodegenerative diseases [218, 219]. There are however limitations in assessing microglial activation *in-vivo* using the PK11195 ligand. Issues regarding a lack of specificity and limitations in crossing the BBB have ushered in testing of second generation TSPO ligands, the accuracy of which remain to be proven [218, 220].

### 1.5.5 Post-mortem evidence

Activated microglia have been found to be a feature of normal ageing [221-223] and there is now a wealth of post-mortem evidence that they are involved in the aetiology and progression of AD [224-228]. Early studies by McGeer et al. showed evidence of large numbers of activated microglia (which were HLA-DR-positive) in the cortex of human AD cases [172, 229], with these microglia found to be clustered around Aβ plaques. In addition, activated microglia have been associated with NFT-bearing neurons in AD [226]. The role of FcγR in AD is particularly interesting. Activation of these receptors on microglia are known to trigger phagocytic activity, with post-mortem evidence showing that they are especially expressed on microglia around Aβ plaques in AD [230], supporting a role for phagocytic microglial response to protein deposition.

Several studies conducted in Southampton have examined microglial phenotype in AD using post-mortem brain tissue. These studies have shown functionally different microglial populations in the AD brain compared with controls, and also shown that CD68-positive microglia were increased in areas of significant Aβ pathology in AD cases immunised against amyloid [231, 232]. This supports the principle that microglia adopt different phenotypes depending on their micro-environment and in this case, that microglia play a key role in the phagocytic removal of Aβ pathology following immunisation against amyloid. Indeed, a further study coordinated in Southampton showed that the presence of dementia in post-mortem cases with AD pathology was

positively associated with CD68 and CD64, both markers of microglial phagocytic activity [174].

A systematic review by Hopperton et al. [169] examined 113 studies that quantified microglial markers in post-mortem human brain tissue from subjects with AD and aged controls. The three most commonly investigated markers, in decreasing order of the number of studies that examined them, were Iba1, HLA-DR and CD68. The authors concluded that a number of microglial markers are significantly increased in the AD brain compared with controls, including HLA-DR and CD68, and that this increase is likely to be attributable to increased microglial activation rather than an increase in absolute cell numbers.

As well as being observed in control brains in small numbers, T lymphocytes have been shown to be more numerous in AD brains. This finding was demonstrated in an observational study of 60 cases using immunostained human post-mortem brain tissue [233]. The increased presence of T lymphocytes in AD brains is thought to result from inflammation and cerebrovascular disease causing BBB damage [234], supporting the hypothesis that there is likely to be interaction between the peripheral adaptive and cerebral innate immune system in AD.

Lastly, the role of microglial priming was discussed in section 1.4.4 and offers an insight into possible mechanisms of communication between the peripheral immune system and neurodegeneration. One theory is that systemic infections or injury may accelerate neuroinflammation and neurodegeneration in AD. The role that systemic inflammation plays in AD will now be reviewed.

### 1.5.6 Body fluid biomarker evidence

Acute systemic infections and chronic inflammation have been implicated in the progression of several neurodegenerative diseases, including AD. There is much evidence that in diseases such as AD, where there is a chronic innate immune response in the brain, systemic inflammation can drive neurodegeneration and exacerbate clinical symptoms [168]. A number of studies have shown differences in inflammatory biomarkers in the blood and CSF of patients with AD compared to controls.

A meta-analysis of 40 studies is supportive of differences in blood markers of inflammation in AD subjects compared with controls [235], with significant increases in pro-inflammatory cytokines TNFα, IL6, IL1β, IL12 and IL18, as well as an increase in the anti-inflammatory cytokine TGFβ, but no change in IL4, IL8, IL10, IFγ or C-reactive protein (CRP). Interestingly, circulating levels of TNFα and IL6 have also been associated with baseline cognitive scores and increased rates of cognitive decline in AD [236, 237]. Longitudinal studies have shown that plasma levels of inflammatory cytokines such as CRP and IL6 were elevated up to five years before the clinical onset of dementia in AD [238, 239]. Moreover, raised CRP in midlife has been associated with a threefold increased risk of developing AD up to 25 years later [240]. Studies examining cytokine concentrations in the CSF of patients with MCI have shown that increased TNFα and decreased TGFβ are risk factors for accelerated cognitive decline and conversion of MCI to AD [241, 242]. These studies suggest that peripheral inflammation may pre-date and possibly even play a role in the onset of AD.

Changes in peripheral blood mononuclear cells (PBMC), including lymphocytes, have been demonstrated in AD. Peripheral CD4 T cell subsets, but not CD8 T cells, have been shown to be increased in AD compared to controls [243]. However, another group has shown decreased CD8 T cells in AD compared with controls [244], whilst yet another group showed both increased CD4 T cells and decreased CD8 T cells [245]. It is noteworthy that the findings above have not been replicated by other groups more recently, who have shown no significant differences in CD4 or CD8 subsets in AD compared with controls [246, 247]. Furthermore, the CD8 T cell subset has been shown to be reduced in not only AD, but in several other causes of dementia, indicating that T cell changes may not be specific for AD [248].

An article reviewing lymphocyte population changes in AD, measured by flow cytometry, demonstrated evidence for a switch from naïve T cells towards memory T cells in AD, indicating that the adaptive immune system in AD may be subject to persistent antigenic challenge [249]. In addition to T lymphocytes, B cell populations have also been examined in AD. Studies have shown either decreased populations of the CD19+ B cell subset in AD compared with controls [247], or no difference [250].

PBMC can be stimulated *ex-vivo* to study the profile of cytokines produced post-stimulation. Several groups have shown that PBMC stimulated with lipopolysaccharide (LPS) in order to mimic bacterial infection, or with the mitogenic agent phytohemagglutinin (PHA), produce elevated concentrations of pro-inflammatory cytokines (e.g. IL1β, IL6 and TNFα) in AD subjects compared with controls [245, 251, 252]. Ragweed mitogen is also a mitogenic agent and stimulation of PBMC with this has been shown to down-regulate CD69 (a marker of cell cycle activity) in AD [253], a response which has recently been proposed as a biomarker to differentiate between PD and AD [254]. In addition, the levels of plasma soluble CD40L (sCD40L), which modulates activation of antigen presentation cells, have been observed to correlate with disease severity and Aβ levels [255-258], and to precede the development of AD [259].

Several groups have suggested that lymphocyte biomarkers could be used to aid early diagnosis of patients with AD [260-262] but all have expressed concern that the standardisation and validation of findings is not yet satisfactory. There may be several reasons for the lack of consistent findings with regards to lymphocyte biomarkers for AD. Relevant factors include differences between studies in terms of sample size, study population demographics and differences in methodologies between groups [249]. Overall there are many papers proposing lymphocyte changes in dementia but there is currently a lack of reproducible data, which precludes the development of a reliable biomarker for AD. Of equal promise, but suffering with the same issues regarding reproducibility of results, is the identification of cytokine biomarkers for AD. Chitinase-3-like-1 (CHI3L1, also known as YKL40) has been proposed as a novel CSF and plasma biomarker for mild AD [263, 264]. In addition, CSF concentration of soluble TREM2 has also been found to be elevated in AD [265], further supporting the possibility of developing an inflammatory-based biomarker for AD. It may be that a combination of cytokine and lymphocyte markers are required to produce a robust biomarker for AD.

### 1.5.7 Anti-inflammatory treatments

Despite the growing evidence base supporting a role for inflammation in the aetiology and progression of AD, there has been little success in targeting the immune system as a treatment for the condition.

Epidemiological studies showing the protective effects of non-steroidal anti-inflammatory drugs (NSAIDs) raised hope in this area. Indeed, several large prospective studies have shown decreased incidence of AD when participants have been taking NSAIDs. The Baltimore Longitudinal Study of Aging included 1,686 participants and showed the relative risk of AD decreased with increasing duration of NSAID use [266]. A large case-control study in the US, with 49,349 cases and 196,850 controls, showed that the odds ratio for AD amongst NSAID users was 0.76 for >5 years use [267]. In addition, a recent study showed that users of disease modifying anti-rheumatic drugs for rheumatoid arthritis were at reduced risk of dementia (hazard ratio of 0.60) [268].

Despite these promising epidemiological studies, several large randomised controlled trials have failed to show any benefits for anti-inflammatory drugs such as aspirin, naproxen and prednisolone [269-272]. In fact, some studies have shown that use of certain NSAIDs may even increase risk of developing AD or worsen cognitive decline in established AD [273, 274]. Follow-up of participants in one of those studies, the ADAPT trial, revealed that the harm caused by NSAIDs was specific to people in the latter stages of AD and that treatment of asymptomatic individuals reduced AD incidence, but only with at least 2-3 years of treatment [275]. Overall it is likely that benefits of anti-inflammatory medication for AD may require specific targeting of certain inflammatory pathways at a pre-symptomatic stage of disease and for a significant length of time.

Most promisingly, one study performed in Southampton examined tolerability and clinical outcomes in patients with AD treated with Etanercept, a TNFα inhibitor, versus controls. It found trends for improvements in cognition (measured by MMSE) and behavioural symptoms (measured with the Neuropsychiatric inventory – NPI) in the treatment group, although these did not reach statistical significance once an intention to treat analysis was carried

out [276]. Since the trial was small (n=41) and only over a 6 month period, further studies are warranted to confirm this encouraging data.

## 1.6 Inflammation in DLB

In contrast to the volume of literature published on inflammation in AD, there has been significantly less research into the role of inflammation in DLB. Due to the paucity of DLB-specific data, much of what is presented below comes from studies investigating inflammation in other LBD such as PD. Indeed there have been numerous review articles summarising the extensive literature on the role of inflammation in PD [54, 277-280], some of which will be appraised below. However, caution should be applied when extrapolating findings from other LBD to DLB, at least until studies specific to DLB have been published.

### 1.6.1 Delirium

Possibly the most intriguing research in this area is the link between DLB and delirium. Delirium is a common condition in the elderly, often caused by infections. It has been shown that 25% of DLB cases had at least one previous reported episode of suspected delirium compared with just 7% of AD cases [281]. Indeed, one of several proposed variants of prodromal DLB is a “delirium onset DLB”, characterised by the presence of delirium as a key presenting feature [282].

A recent review article has summarised the close relationship between DLB and delirium, with similarities found in the pattern of cognitive impairment (e.g. impaired attention) and symptom profile (e.g. fluctuations and hallucinations), along with significant overlaps in neurochemical cholinergic deficits [283]. However, significant correlations in imaging, neuropathology and inflammation were not possible, primarily due to the paucity of research in the two conditions. Since infection is a common cause of delirium, the overlap between this condition and DLB offers merely a suggestion of a role for inflammation in DLB.

### 1.6.2 Pre-clinical evidence

The primary component of LB, α-syn, has been consistently shown to induce microglial activation in both cell culture studies [284], and in PD mouse models [285-288]. Aggregates of α-syn have also been shown to be a chemoattractant for microglia in cell culture, through binding of the microglial marker CD11b [289]. Furthermore, pellets of slow-release IL1β have been implanted in rodent brains with increased messenger ribonucleic acid (mRNA) expression of α-syn detected [290], showing that pro-inflammatory cytokines may play a role in the development of neuropathology. In addition, research with PD mouse models has shown T cell infiltration into substantia nigra, raising the possibility of a role for the adaptive immune system in the pathogenesis of the disease [291]. Other work has shown the induction of MHCII positive microglia by α-syn in a mouse model, which does not occur in MHCII knock-out mice [292]. These studies support the notion that microglia are activated by the presence of α-syn *in-vitro* and in animal models.

Despite the evidence presented above, the precise mechanism of activation of microglia by α-syn in humans remains unclear, particularly as deposition of α-syn occurs intracellularly, thus limiting its direct exposure to microglia. The window of exposure to α-syn fibrils may be limited to the transient movement of this pathology to the extracellular space during prion-like propagation between neurons. Choi et al. showed that in cell cultures α-syn aggregates in fact inhibit microglial phagocytosis due to activation of CD32b on microglial cells [293], and that neuronal CD32b functions as a receptor for α-syn fibrils to mediate cell-to-cell transmission of α-syn [294].

There is however contrasting evidence in this area. A noteworthy study examined the role of FcγR in a mouse model of PD that over-expressed α-syn - a model that has been shown to trigger the expression of the transcriptional and inflammatory protein NFκb-p65, which leads to pro-inflammatory signalling by microglia. The authors showed that microglia successfully phagocytosed α-syn in both wild-type and FcγR knockout mice. However, the FcγR knockout mice failed to show increased expression of NFκb-p65 and associated pro-inflammatory signalling was reduced [295]. This showed that α-syn is actively phagocytosed by microglia and that FcγR play a role in the type

of antibody-mediated inflammatory response, with an associated role in pro-inflammatory signalling that could cause damage to healthy tissue.

Therefore, the exact nature of interaction between α-syn and microglia with regards to phagocytosis has not been conclusively demonstrated. It may be that α-syn aggregates play a role in the induction of pro-inflammatory signalling by microglia, but may in fact actively inhibit microglial phagocytosis, possibly hampering the effective clearance of these protein deposits in PD. In turn, this could lead to further propagation of pathology between neurons and the spread of pathology through the brain.

The anti-inflammatory drug Montelukast, used in the treatment of asthma, has been shown to reduce neuroinflammation and improve cognition in a mouse model that over-expresses α-syn [296]. However, full appraisal of this study is not possible as only the abstract is available to view currently. More broadly, there is extensive data showing the neuroprotective effects of NSAIDs in animal models of PD [297].

Another noteworthy study examined the effect of LPS injections into the substantia nigra of mice. The authors observed microglial activation with increased IL1β and TNFα, along with impairment of motor function. However, when the same procedure was tested in IL1 knock-out mice, the extent of behavioural deficits was significantly less prominent than those found in the wild-type mice [298], suggesting a possible role for IL1 in microglial activation and symptomatology in PD. However, there were several limitations in this study, with the control mice being wild-type and not a PD model, along with a lack of detailed analysis of microglial phenotype.

Many PD mouse models lack the chronicity of pathology that occurs in humans (with degeneration of the nigrostriatal pathway occurring in days, not years) and many also lack the presence of LB, meaning their applicability to human PD and DLB is extremely limited [299].

### 1.6.3 Genetic evidence

In contrast to AD, there has only been one major GWAS in DLB published to date. Interestingly this failed to reveal any genes associated with inflammation, although the authors noted that their sample size was relatively small for a

typical GWAS [38]. It appears that larger sample sizes will be required in order to detect loci associated with inflammation, with work underway through international collaboration to secure larger numbers of samples from DLB patients.

Notably, a small neuropathology-based genetic study investigating LRP as a phenotypic trait found an association between LRP and HLA-DP [300], suggesting that antigen presentation may play a particularly important function in LBDs. These results need to be confirmed as the study had limited power and did not specifically include patients with a clinical diagnosis of DLB.

There is extensive genetic evidence supporting a role for inflammation in PD, with numerous studies showing polymorphisms in several inflammation-related genes as being risk factors for the disease, including *TREM2, IL1β, TNFα, LRRK2 and HLA-DR* [301-305]. However, there does not appear to be a role for *APOE* polymorphisms in the aetiology of PD [306], contrary to findings in both AD and DLB.

### 1.6.4 Epidemiological evidence

During a literature search, no relevant work was identified that examined prospective epidemiological data relating to inflammation in DLB. However, similar to AD, studies have shown evidence of reduced risk of PD with use of NSAIDs, particularly with usage for longer than 2 years [307, 308]. However, more recently two meta-analyses have failed to find an effect for NSAIDs as a drug class in reducing risk of developing PD, but have found reduced risk for people taking Ibuprofen specifically [309, 310].

One recent study has identified reduced incidence of PD amongst people diagnosed with inflammatory bowel disease who were also taking anti-TNF medication; in fact the incidence rate was lower than in the general population [311]. This suggests that not only could anti-TNF medication reduce risk of PD in those with inflammatory bowel disease, but that it could also prove beneficial in the general population.

A large primary care, retrospective cohort study examined whether NSAID use was associated with diagnosis of AD, DLB or VaD. It showed no association between NSAID use and risk of AD or DLB, but an increased risk for VaD [312].

Whilst this study was very large (n=31,083), it should be noted that cases were selected retrospectively from GP records using medical diagnostic codes from 1992-2014. Therefore this data may have been subject to significant errors, not only by the considerable challenges of accurately recording dementia in primary care but also by changes in diagnostic guidelines and clinical practice over time.

Strikingly, and in contrast to the evidence above that suggests a possible role for NSAIDs in reducing risk of developing PD, many of the known chronic inflammatory conditions that are risk factors for AD were not identified in an article reviewing the epidemiology of PD [313].

### 1.6.5 Imaging evidence

One pilot study has examined PK11195 signal using PET imaging in six cases of DLB, showing increased signal in cortical and subcortical areas compared with controls. This was in contrast to the PD group, which showed increased signal only in subcortical areas [314], a pattern which is consistent with localisation of disease pathology and symptomatology. The age of the control group was significantly younger than the DLB group, but this was corrected for during statistical analysis. It is noteworthy that all DLB and PD cases included in this study were imaged within one year of symptom onset and hence whilst the results would support microglial activation in mild or early DLB, it is not possible to exclude the pattern of neuroinflammation changing as the disease progresses.

More recently, one abstract has been published of a study examining PK11195 PET imaging in 11 cases of DLB. This study appears to show increased microglial activation in the basal ganglia and occipital lobe in DLB compared to controls, with PK11195 signal correlating positively with cognitive performance but no correlation with disease duration [315]. It is of note that the two cerebral regions that were identified in this study are regions known to be affected in DLB. The correlation with cognition suggests that microglial activation may be particularly prominent in milder stages when patients may be performing better on cognitive testing; however the lack of correlation with disease duration fails to support this. Disease duration is a challenging variable to study as it normally relates to symptom duration rather than

duration of neuropathology, the latter of which probably has more relevance but is difficult to study *in-vivo*. Another explanation of these results could be that microglial activation, as measured by PK11195 signal, could in fact be neuroprotective and play a role in maintaining cognition in DLB. Alternatively, the increased PK11195 may not be measuring microglial activation, but instead astrocytic activation, possibly occurring in order to support synaptic transmission in DLB. Similar to the study above, another abstract has been published examining a second generation TSPO ligand, DPA713, in 10 cases of DLB and found increased binding across the cortex in DLB [316]. It should be noted that neither of these two studies had been published at the time of writing, precluding thorough appraisal of methodology and data.

The extent of neuroinflammation using imaging ligands in PD and PDD has been comprehensively reviewed by Surendranathan et al. [317]. Of particular note are two studies – one which showed increased PK11195 signal in the basal ganglia, pons, frontal and temporal regions in PD [318], and another showing increased signal in the striatum and cortical areas in PDD [319]. It has also been shown that microglial activation, measured by PK11195 PET signal, inversely correlates with glucose metabolism in PD, suggesting that cortical inflammation and neuronal dysfunction are related [320]. Overall it appears that microglial activation, as measured by PK11195 binding, is a significant feature in both PD and PDD.

It should be emphasised that there is still uncertainty as to precisely what increased PK11195 PET signal indicates, despite the authors of the studies above concluding that it represents microglial activation. PK11195 has low brain permeability and a lack of specificity to microglial binding. Other immune cells, include astrocytes, have been shown to also take up the PK11195 tracer [219, 220]. Therefore the exact type and level of activation of microglia deduced from these imaging studies has yet to be confirmed. As stated previously, testing of second generation TSPO ligands are underway, which may prove to be more specific as markers of microglial activation.

### 1.6.6 Post-mortem evidence

Microglia have been observed to be activated around LRP in PD post-mortem brain tissue compared with controls [229, 321-323]. Similar findings have also

been published in DLB, with HLA-DR positive microglia shown to extend their processes to degenerating neurons containing LB [324, 325].

The extent of microglial activation in DLB has been the subject of debate, with several small neuropathology studies demonstrating inconsistent findings. Mackenzie reported greater numbers of activated microglia (immunostained for HLA-DR) in DLB (n=5) and AD brains compared with healthy controls, with the number of microglia positively correlating with numbers of LB in several brain regions [326]. In contrast, and in the same year, Shepherd et al. found that the number of activated microglia (also immunostained for HLA-DR) in DLB (n=8) was not significantly different to that found in controls, and significantly fewer than in AD [327]. The authors of this study suggested that neuroinflammation may not play a prominent role in DLB and stated that since ptau tangles may drive the neuroinflammatory process in AD, their relative scarcity in DLB could explain the lack of neuroinflammation they found in DLB. Subsequently, in a letter to the editor of Arch Neurol, Mackenzie (author of the first study) stated that these differences could be explained by two methodological factors. Firstly, Mackenzie proposed that his group of DLB cases were selected to have minimal amyloid plaque pathology and were therefore “pure DLB”. Secondly, he stated that his method of quantifying microglial activation by sampling an entire cortical region, rather than selecting an area for maximum cellularity, would provide a more representative estimate on the level of inflammation. Mackenzie stated that these two methodological differences could explain the conflicting results between the two studies [328]. In my opinion, there are potential methodological flaws in the MacKenzie study. There is a lack of information regarding inclusion criteria for DLB cases, which appear to have been selected based on presence of LRP and absence of “senile plaques” using the Consortium to Establish a Registry for Alzheimer disease (CERAD) criteria, but with no mention of ptau neurofibrillary tangle pathology. If these DLB cases had intermediate or even high Braak stages, this could be a potentially significant confounder.

In addition to the two studies above, there have been a number of others published. A Japanese group reported that the number of HLA-DR-positive microglia in the hippocampus of a small number of DLB cases (n=5) was significantly higher than found in controls [329]. Another recent study found

no difference in Iba1 staining between DLB and controls, but higher CD68 staining in DLB (n=14) [330]. Additionally, a further study examined activated microglia, using Iba1 and CD68, in the hippocampus and found no difference in numbers in the DLB group (n=12) compared with either controls or AD [331]. Lastly, yet another study examined protein immunoreactivity for Iba1 and HLA-DR in the pulvinar and found no difference in DLB (n=14) compared with controls, although it did show increased astrogliosis in DLB [332]. In PDD, no change in microglial numbers were found in the neocortex (n=15) compared with controls. The PDD cases included in this study had minimal co-existing AD pathology [333].

One study has reported on the expression of cytokines in the neocortex of patients with DLB using immunohistochemistry. The authors reported increased expression of IL1α and TNFα, by measuring positively stained microglial cells (using double immunohistochemistry with HLA-DR) in several cortical areas in both DLB and AD compared to controls, with close regional associations with LRP in the DLB cases [334]. The results suggest that overexpression of these cytokines may play a role in interacting with LRP during neurodegeneration. However, the images presented in this study to demonstrate cytokine immunoreactivity are not clear or conclusive, and it is not apparent whether the cells presented in the paper are microglia, astrocytes or in some cases even cells at all.

One interesting study has shown the potential involvement of the adaptive immune system in PD. Orr et al. found increased IgG positive neurons in the substantia nigra in PD, with the number positively correlating with the number of HLA-DR positive microglia. There was also increased expression of CD64 on these activated microglia, which were shown to contain pigment granules consistent with completed phagocytosis of the IgG-positive pigmented neurons [335]. The potential involvement of IgG in a localised region of the brain, the area of which is consistent with PD pathology, suggests a role for the adaptive immune system in the pathogenesis of PD.

When reviewing the literature presented above as a whole, there does not appear to be a clear picture of the role of microglia in DLB. Many of these studies had common limitations. Cases were often selected based on neuropathological features alone and did not include clinical history. A

maximum of one or two markers of microglial phenotype were often used. In addition, many studies used microglial counts as their outcome measure rather than examining phenotype. All studies suffered with small sample sizes, especially for the DLB groups, meaning that some may have been under-powered to detect a significant difference. It should also be noted that many of the studies reviewed above took place prior to consensus international criteria for the neuropathological diagnosis of DLB, further diluting their applicability to DLB populations diagnosed today. Overall there appears to be conflicting evidence regarding the role of microglia in DLB with a lack of comprehensive data looking at a variety of markers of microglial phenotype in a large cohort of DLB brains.

### 1.6.7 Body fluid biomarker evidence

There is a lack of published research examining peripheral inflammation in DLB, with only a handful of papers found in a search of the literature. Indeed, a recent systematic review of the role of peripheral cytokines and CRP in LBD comprehensively examined the literature for studies investigating blood and CSF cytokine changes, but found just two studies that involved patients with DLB [336].

In DLB, Clough et al. showed that increasing serum levels of the pro-inflammatory cytokines IL6 and TNFα were associated with worsening neuropsychiatric symptoms and worsening cognition, respectively [337]. However, the study included patients with either possible or probable DLB, meaning limited applicability of the findings to a population wholly diagnosed with probable DLB, as per the international consensus criteria.

The only other study examining blood markers of inflammation in DLB was published by King et al. in 2018 [338]. This study examined plasma samples of 37 DLB patients and compared them to AD and controls. There were also two MCI groups (MCI-DLB and MCI-AD), the inclusion of which made this study particularly noteworthy. The authors showed significantly higher IL1β, IL4, IL2 and IL10 concentration in both MCI groups, with no difference found between DLB and controls, or in fact between AD and controls. This strongly supported a role for increased peripheral inflammation at the prodromal stage of disease pathogenesis, which then disappears with disease progression in both DLB and

AD. Indeed, there is now work underway to develop the concept of MCI-DLB, or prodromal DLB [339], and it has been suggested that there will be a greater potential for intervention with drugs targeting inflammation early in pathogenesis if the reliable identification of prodromal DLB can be improved [340]. This theory is however complicated by further data from the King et al. study, which showed that when the DLB and AD groups were combined, lower levels of IL1β, IL2 and IL4, and higher levels of IL6 and TNFα, were positively correlated with greater severity of cognitive impairment. When assessing for correlations with worsening Parkinsonism in DLB only, the same associations were found. These data appear to show a role for certain inflammatory cytokines early in the disease process in both DLB and AD that then changes with increased symptom severity, suggesting a complex interplay between cytokine concentration and specific symptoms as dementia progresses.

Looking at CSF cytokine levels, Wennstrom et al. showed that IL6 concentration was significantly lower in 29 DLB patients compared to both AD and controls [341]. Interestingly, this study also demonstrated that CSF IL6 levels were negatively correlated with MMSE in DLB. This could lead one to believe that IL6 may play an increasing role as cognitive impairment worsens in DLB, but it should be emphasised that overall this inflammatory marker was shown to be reduced in DLB compared with controls. In contrast with the last paper, Gomez-Tortosa et al. found that CSF levels of IL6 in a group of 25 DLB patients were not significantly different from AD [342]. Another study investigated the inflammatory marker CHI3L1 in the CSF of AD and DLB subjects and found significantly lower levels of the protein in DLB compared to AD, with concentrations in the DLB group similar to the control group [343]. These three studies fail to convincingly demonstrate a clear role for CSF markers of inflammation in DLB, and clearly much more work is required in this area in order to form a decisive conclusion.

One notable study has examined PBMC in DLB and found the presence of small subgroups of peripheral T cells responsive to Aβ in AD subjects but not in DLB [344], with the authors postulating their use as a potential diagnostic biomarker. This study appears to show changes in how the peripheral adaptive immune system reacts to the presence of cerebral neuropathology, raising the possibility of an altered adaptive immune system profile in DLB. No other

studies examining PBMC populations in DLB were identified in a review of the literature.

In contrast to the DLB studies presented above, there has been extensive research investigating the role of systemic inflammation in PD, and to a lesser extent in PDD. A review of the literature shows that PD patients have higher serum levels of IL1β, IL6, IL8, TNFα, IFNγ and RANTES, when compared with controls [345-353]. These findings were largely confirmed in a recent meta-analysis showing increased blood levels of IL1β, IL2, IL6, IL10, CRP and RANTES in PD [354]. The role of the chemokine RANTES is particularly interesting as it is a strong chemoattractant for lymphocytes, suggesting a role for activation of the adaptive immune system in PD. Another recent meta-analysis examined studies including patients with both PD and PDD, and concluded that there was a general increase in blood IL1β, IL6, IL10 and TNFα compared with controls, but that nearly an equal number of studies showed non-significant differences in each of these cytokines [336]. IL1β was particularly highlighted by the authors as being elevated in all studies examining blood concentration of this cytokine in PD and PDD. The overall picture appears to be one of increased concentrations of pro-inflammatory cytokines, and the anti-inflammatory cytokine IL10, in PD and PDD compared with controls. However, there are discrepancies between the results of many original studies, with numerous studies showing no statistically significant alterations in cytokine concentration in PD or PDD.

When examining for associations between cytokine concentrations and clinical measures, several noteworthy associations have been identified in PD. Serum IL6 levels have been shown to be raised prior to motor symptom onset and also been shown to significantly increase the risk of developing PD [355], suggesting that peripheral inflammation may play a significant role in very early neurodegeneration in PD. Furthermore, increased serum and LPS-induced PBMC production of IL1β, IL8, IFNγ, TNFα and RANTES have been shown to be higher in PD compared with controls, and the levels of those cytokines correlating positively with disease severity, as measured by the Unified Parkinson's disease rating scale (UPDRS) motor examination score [356]. In addition, serum concentrations of IL1β, IL2, IL10 and TNFα were found to be higher in PD compared with controls. Interestingly, when the PD group was

dichotomised into “higher” and “lower” pro-inflammatory groups, the “higher” pro-inflammatory group showed cytokine concentrations that were associated with more rapid motor symptom progression and lower MMSE score [357]. These data show that the peripheral immune system is modified in PD and that it may be a driver for worsening motor symptoms.

CSF markers of inflammation in PD have also been thoroughly examined. Studies have shown that PD patients have higher CSF levels of TNFα and IL6 compared with controls [358, 359]. Furthermore, CSF IL6 concentration has been found to be significantly higher in PDD compared to PD alone [360]. CSF IL6 concentration has also been shown to be inversely correlated with UPDRS motor score [361], suggesting a role early in the disease process. Therefore, CSF IL6 may peak both early in PD but also later in PDD.

Lastly, PBMC subset changes have also been well documented in PD. Compared with controls, B lymphocytes in PD have been shown to be reduced along with the percentage of CD4 T cells, the latter mainly due to the loss of naïve helper T cells [362]. Furthermore, this year another group published that CD3+ lymphocytes and activated CD4+ lymphocytes were significantly lower in PD compared with controls, but there was no difference in B cell or CD8+ T cells cell populations [363]. Lastly, another group showed reduced CD4+ T cells and B cells in PD compared with controls [364]. These PBMC studies show reduced lymphocyte subset populations, particularly of helper T cells, that indicates a modification of adaptive immunity in PD.

### 1.6.8 Anti-inflammatory treatments

As previously stated, the regular use of NSAIDs and in particular Ibuprofen, has been shown to reduce the incidence of PD in several epidemiological studies [307-310]. However, there have been no randomised controlled trials performed to date examining the effect of anti-inflammatory drugs as treatments for established PD, PDD or indeed DLB.

Interestingly, several clinical trials are underway to trial the treatment of PD with active or passive immunotherapy, targeting the removal of α-syn from the brain or by promoting the prevention of pathology propagation, as reviewed by

George et al. [92]. However, data from phase 2 clinical trials in this area have yet to be published. In addition, immunotherapy has yet to be trialled in DLB.

## 1.7 Scope for this project

DLB is a challenging clinical diagnosis to make. This is unsurprising as the clinical features, genetics, neuropathology and neurochemistry of the disease overlaps with both AD and PD. Delays in achieving the correct diagnosis can cause the use of potentially inappropriate treatments to be used, such as the use of antipsychotics in DLB. Clinical diagnosis of DLB is based on assessment of symptomatology, cognitive testing and sometimes expensive brain imaging. There are, however, currently no disease modifying treatments available for DLB, or indeed any other cause of dementia. There is also no well-established or proven aetiological theory explaining the cause of DLB.

Over recent years there has been an increasing body of evidence supporting a role for inflammation in the aetiology and progression of AD and PD. This has led to the testing of drugs targeting the peripheral immune system in early AD as a novel treatment. In contrast, there has been little research in the field of inflammation in DLB. Several studies have shown conflicting results as to whether markers of the immune system are altered in DLB. The handful of neuropathology studies, in particular, have only examined a couple of microglial markers in the brain and the number of DLB cases studied has generally been few. Furthermore, there have been no large studies examining lymphocyte sub-populations or whole blood stimulated cytokine levels in DLB to date.

This project aims to investigate these areas by using a clinical cohort of DLB patients to investigate peripheral inflammation, along with a large cohort of post-mortem brain tissue to investigate cerebral inflammation in DLB. The expectation is to provide new information regarding the role of inflammation in DLB. Improving our knowledge of the aetiology of DLB will help to inform new immunity-related therapeutic targets and could also guide the development of biomarkers that increase diagnostic accuracy.

# Chapter 2: Hypothesis, aims and experimental approach

## 2.1 Hypothesis and aims

The following hypothesis is proposed in order to address the role of inflammation in DLB:

> ***Systemic and cerebral inflammation will show a profile specific to DLB compared with aged controls and/or AD, and will be associated with the clinical features and/or neuropathology of the disease.***

In order to investigate this hypothesis, the following aims will be explored:

1. To define the phenotype of peripheral immune cells and serum markers of inflammation in DLB, AD and controls.
2. To determine the microglial immunophenotype in DLB compared to controls.
3. To determine if any markers of inflammation, cerebral or peripheral, are associated with the neuropathological and/or clinical features of DLB.

## 2.2 Experimental approach

In order to achieve the aims above, this project will be split into two studies: 1) a clinical study investigating peripheral inflammation, and 2) a neuropathology study investigating cerebral inflammation. Aim three will be addressed in both studies. A brief summary of the experimental approach to the two studies follows.

### 2.2.1 Peripheral inflammation in Dementia with Lewy bodies and Alzheimer's disease (SILAD)

The clinical study will be a cross-sectional observational trial recruiting patients with a clinical diagnosis of probable DLB, patients with probable AD and aged controls. Clinical data will be obtained during a single study visit, along with various cognitive and neuropsychiatric assessment scores. Blood samples will be taken for genotyping, investigation of peripheral immune cells and cytokine concentrations. Peripheral blood mononuclear cell (PBMC) stimulation studies will be performed to examine levels of stimulated cytokines. Markers of peripheral inflammation will be correlated with clinical features and neuropsychiatric assessment scores in DLB and AD.

Methods, results and conclusions from this study are presented in Chapter 3.

### 2.2.2 Cerebral inflammation in Dementia with Lewy bodies (CIDL)

The post-mortem study will utilise immunohistochemistry to investigate a number of markers of pathology and inflammation in human brain tissue. Cases of DLB and matched controls will be selected from UK brain banks. Various markers associated with specific microglial functions will be used to determine the phenotype of these immune cells. Digital images will be taken of immunostained tissue and a percent protein load obtained from image analysis. Protein loads of microglial markers will be correlated with those of neuropathology markers in both DLB and control groups. A separate AD group will not be included as this work has already been completed using the same protocols within the Boche laboratory, and it was felt that duplicating this work would not be appropriate. However, data obtained from this AD work will be used for comparison with the DLB cohort.

Methods, results and conclusions from this study are presented in Chapter 4.

# Chapter 3: Peripheral inflammation in DLB and AD

There is now a significant body of evidence supporting the role of peripheral inflammation in the aetiology and progression of AD and PD. In contrast, there have been only a few small studies that have investigated peripheral inflammation in DLB. This chapter will present methods and results from a cross-sectional observational clinical study, named SILAD (**S**ystemic **I**nflammation in dementia with **L**ewy bodies and **A**lzheimer's **D**isease).

## 3.1 Methods

### 3.1.1 Power calculations

Power for the PBMC stimulation study was based on a study by Reale et al. in PD [356] that showed serum TNFα concentration was increased by a ratio of 1.63 in PD. The study reported a mean of 19.3 (s.d. 7.3) pg/ml in PD, compared with the control mean of 11.8 (s.d. 2.8) pg/ml. Allowing for Bonferroni correction for 2 stimuli (LPS and PHA), 2 comparisons (DLB c.f. AD, DLB c.f. controls) and 10 cytokines, an alpha of 0.00125 (2 tailed) was agreed. Assuming the higher standard deviation (7.3 pg/ml), 40 patients in each group gave 90% power to show a mean difference between groups of 7.5 pg/ml. Assuming a mean standard deviation (5.05 pg/ml), 21 patients in each group gave 90% power to show a mean difference between group of 7.5 pg/ml.

Power for the serum cytokine analysis was based on the same study in PD by Reale et al. that showed the key pro-inflammatory cytokine TNFα was more than twofold greater in PD than in controls [356]. The study reported a mean of 14.9 pg/ml (s.d. 3.9pg/ml) in PD, compared with a mean of 5.1pg/ml (s.d. 1.7pg/ml) in controls. Allowing for Bonferroni correction for 10 serum cytokine measures and 2 comparisons (DLB c.f. AD, DLB c.f. controls), an alpha of

0.0025 (2 tailed) was agreed. Assuming the higher standard deviation (3.9 pg/ml), 8 patients in each group gave 90% power to show a mean difference between groups of 9.8 pg/ml. Assuming a mean standard deviation (2.8pg/ml), 5 patients in each group gave 90% power to show a mean difference between group of 7.5pg/ml.

Allowance for dropout was not required due to the cross-sectional nature of the study. However, given potential heterogeneity in clinical samples, and as I planned to investigate correlations between inflammatory markers and clinical outcomes in the DLB group, a larger sample was required. It was therefore agreed to aim for recruitment of 40 participants in each group (DLB, AD and controls) and accept that even if this was not achieved the study should still be adequately powered to detect significant differences and associations between key variables.

### 3.1.2 Ethics

Study material was prepared by me under the supervision of Prof Clive Holmes. The study was approved by the National Research Ethics Committee (NREC) London Hampstead (reference 14/LO/1510) in 2014. Local Research and Development (R&D) approval for the study followed in 2015 (Southern Health NHS Foundation Trust). The chief investigator for the study was Prof Clive Holmes.

The Memory Assessment and Research Centre (MARC) at Moorgreen Hospital in Southampton, part of Southern Health NHS Foundation Trust, was originally determined to be the single study site. Subsequently a second site, St Mary's Hospital in Portsmouth (Solent NHS Trust), was added following relevant R&D approval. A non-substantial amendment to the ethics application was made relating to the addition of the second site and also the addition of the Join Dementia Research (JDR) website as a further source of subject recruitment. I was principle investigator and study co-ordinator at the MARC site. Dr Kayode Osanaiye was principle investigator and Mrs Sharon Simpson the study co-ordinator at the Solent site. Site initiation meetings and training for rating scales was provided by myself to all staff at MARC and Solent to ensure compliance with the study protocol. A copy of the ethical committee approval letter for SILAD is shown in Appendix B.

### 3.1.3 Study design

SILAD was an observational, cross-sectional clinical study that took place on the South coast of England. Funding through a Clinical Research Fellowship was received from Alzheimer's Research UK with support from the Lewy Body Society, and sponsorship was provided by the University of Southampton. The research study proposal was designed in consultation with Prof Clive Holmes and the clinical research team at MARC, who have extensive experience in the organisation of observational and interventional trials in dementia.

Several methods were used to try to recruit 40 patients with a clinical diagnosis of probable DLB [9], 40 patients with probable AD [6] and 40 aged controls. The majority of participants were identified from local community mental health team caseloads through referral by their psychiatrist or care coordinator. Other participants were recruited through self-referrals to MARC and via the website JDR. Many control participants were carers of patients with dementia who expressed an interest in taking part in research. All participants were approached either by me (Principal Investigator at MARC), or the senior nurse at the second study site, to explain the study rationale and procedures. Potential DLB and AD participants required a study partner to attend the study visit. Control subjects did not require a study partner.

Interested participants were sent a detailed Participant Information Sheet (PIS) and allowed ample time to consider taking part in the study. Any outstanding questions were addressed at a follow-up telephone contact, and if appropriate an appointment was made for the one-off study visit. A copy of the PIS is shown in Appendix B.

A number of inclusion and exclusion criteria were applied to screen for participants for SILAD, as shown in tables 3.1 (DLB and AD groups) and 3.2 (control group). These contained the exclusion of subjects with acute infections or those receiving medications that could significantly affect inflammatory markers.

**Table 3.1: Inclusion/exclusion criteria for DLB and AD groups in SILAD**

| **Inclusion criteria – DLB and AD groups** |
|---|
| Subject must be aged between 50 and 100 years |
| Subject must have a reliable study partner |
| The subject must meet the NINCDS-ADRDA criteria for probable Alzheimer's disease or the McKeith criteria for probable Dementia with Lewy Bodies |
| The subject must have adequate visual and auditory acuity to allow cognitive testing to be performed |
| The subject and study partner are willing and able to participate for the single visit |
| Subject must be fluent in English language |
| MoCA score at baseline less than 26 (discretion of the Chief Investigator) |
| Signed informed consent by subject prior to the initiation of any study-specific procedure |
| **Exclusion criteria – DLB and AD groups** |
| Refusal to provide informed consent |
| Lack of capacity to provide informed consent |
| Unlikely to cooperate in the study or not be able to follow study instructions |
| Participation in another research study with administration of any investigational drug at time of enrolment |
| The subject's health is not adequate to comply with study procedures, as ascertained by review of their screening medical history |
| Current alcohol >35 units per week for men, or > 28 units per week for women, or drug abuse at the discretion of the Chief Investigator |
| Any psychiatric diagnosis that may interfere with the subject's ability to perform study assessments |
| Subjects taking major modifiers of the immune system including corticosteroids and TNF inhibitors, left to the Chief Investigator's judgment |

**Table 3.2: Inclusion/exclusion criteria for control group in SILAD**

| Inclusion criteria – Control group |
|---|
| Subject must be aged between 50 and 100 years |
| The subject must have adequate visual and auditory acuity to allow cognitive testing to be performed |
| The subject is willing and able to participate for the single visit |
| Subject must be fluent in English language |
| MoCA score at baseline equal to or greater than 26 points |
| Signed informed consent by subject prior to the initiation of any study-specific procedure |
| **Exclusion criteria – Control group** |
| Refusal to provide informed consent |
| Lack of capacity to provide informed consent |
| Unlikely to cooperate in the study or not be able to follow study instructions |
| Participation in another research study with administration of any investigational drug at time of enrolment |
| Any previous or current medical condition that may impact on cognitive performance, left to the Principal Investigator's judgment |
| The subject's health is not adequate to comply with study procedures, as ascertained by review of their screening medical history |
| Current alcohol >35 units per week for men, or > 28 units per week for women, or drug abuse at the discretion of the Chief Investigator |
| Subjects taking cholinesterase inhibitor medication |
| Any psychiatric diagnosis that may interfere with the subject's ability to perform study assessments |
| Subjects taking major modifiers of the immune system including corticosteroids and TNF inhibitors, left to the Chief Investigator's judgment |

### 3.1.4 Study visit

The majority of study visits were undertaken at one of the two clinical sites, with the remainder performed at the participant's home address. Travel expenses were offered if the participant travelled to the clinical sites, but no other financial benefits were paid to participants or their study partners.

All subjects provided informed consent at the start of the study visit. Following a review of the study protocol, time was allowed to answer any outstanding questions from the participant and their study partner (if applicable). Study consent forms were then signed by the participant and study partner, and countersigned by the investigator.

Following the consent process, the following information was documented: demographic details, information regarding diagnosis of dementia (if applicable), medical history and drug history. Information was specifically gathered regarding any chronic inflammatory conditions in the past medical history. Height and weight of all participants was measured. Discrepancies in demographic or clinical data were checked with the study partner and GP practice, when required. Copies of source documentation and consent forms for SILAD are shown in Appendix B.

Next, all participants were subject to cognitive assessment using the Montreal cognitive assessment [115]. Participants in the DLB and AD groups were also subject to further assessments, namely the Free and Cued Selective Reminding Test – Immediate Recall [365], the Clinician Assessment of Fluctuation [366], the Neuropsychiatric Inventory to assess for psychotic and behavioural symptoms [367], the Cornell Depression Score to assess mood [368] and the Unified Parkinson's Disease Rating Scale to assess motor symptoms using a physical examination [369]. Copyright permission for use of each assessment tool was sought by the author (where required). Further details of all assessment tools used are shown in table 3.3.

**Table 3.3: List of neuropsychiatric assessment tools used in SILAD**

| Assessment tool | Description |
|---|---|
| **MoCA**<br>Montreal Cognitive Assessment | Well-validated and widely used tool. It measures 8 domains of cognition: visuospatial/executive function, naming, memory, attention, language, abstraction, delayed recall and orientation. The maximum score is 30. A score above 26 represents normal cognitive function. |
| **FCSRT-IR**<br>Free and Cued Selective Reminding Test – Immediate Recall | Measures episodic memory of 16 items in three rounds. Maximum score for free recall is 48, with the maximum free recall + cued recall also 48. The test is widely used in research settings. |
| **CAF**<br>Clinician Assessment of Fluctuation | Measures fluctuation in alertness and cognition. Two screening questions are asked to the carer and the scores of each (0-4) are multiplied together. 0 indicates no fluctuation and 16 represents continuous fluctuation. |
| **CSDD**<br>Cornell Scale for Depression in Dementia | Assesses for signs and symptoms of major depression in patients with dementia. Semi-structured interview with patient and separately with the informant. Each item is rated for severity on a scale of 0-2. Maximum score is 38. Scores above 10 indicate probable depression. |
| **UPDRS**<br>Unified Parkinson's Disease Rating Scale | Combines elements of a number of scales to produce a tool to assess the severity of, and disability caused by, Parkinson's disease. Sub-scales are:<br>1. Cognition, behaviour and mood (maximum score 16)<br>2. Activities of daily living (maximum score 52)<br>3. Motor examination (maximum score 56)<br>4. Complications of therapy (not used in SILAD)<br>5. Modified Hoehn and Yahr staging (maximum score 5) |
| **NPI**<br>Neuropsychiatric Inventory | Assesses for the presence of behavioural and psychological symptoms of dementia, based on a structured interview with the carer. Assesse 10 domains: delusions, hallucinations, agitation/aggression, depression, anxiety, elation, apathy, disinhibition, irritability/lability, aberrant motor behaviour. Maximum score for each domain is 12 (total 120). Includes a carer stress score, the maximum score for each domain is 5 (total 50). |

### 3.1.5 Blood samples

Blood samples were obtained from each participant and, where possible, these were taken at the same time point (11:00 – 13:00). Listed below are details of how the blood samples were processed and stored.

- Ethylenediaminetetraacetic acid (EDTA) blood tube, 1x 2ml, stood upright then frozen at -80ºC within 1 hour of collection. Samples were sent to our collaborator Dr Jose Bras, University College London, for *APOE* genotyping.
- PAXgene RNA blood tube, 1x 2.5ml, frozen at -20ºC after 2 hours. Then transferred to -80ºC after a further 24 hours. PAXgene samples remain stored for future research into protein expression in DLB and AD.
- Plain, red top blood tube, 2x 10ml, centrifuged at 3000rpm and serum layer removed by pipette. Samples were separated into aliquots (to prevent repeat freeze-thaw cycles) and frozen at -80ºC within 1 hour of collection until batch analysis.
- Heparinised blood tube, 1x 6ml, PBMC were isolated at a laboratory at MARC within 4 hours of phlebotomy, as described in section 3.1.6.

All products isolated from whole blood were stored at -80ºC at MARC, before transfer to -80ºC storage at Clinical Neurosciences, University of Southampton, Southampton General Hospital. All blood samples, and their derivatives, could only be identified using the unique study number allocated to each participant. A log of unique study numbers and participant details was kept securely in the SILAD investigator folder at MARC.

### 3.1.6 Isolation of PBMC

The on-site isolation of PBMC from whole blood required optimisation of methods with support from Dr Yifang Gao at the Wessex Investigational Sciences Hub laboratory (WISH), University of Southampton. The methods are detailed below.

Whole blood collected in a heparinised blood tube was carefully layered onto 6ml of Ficoll using a pipette gun in a laboratory safety cabinet. Being particularly careful not to mix the blood and Ficoll beforehand, this was

centrifuged at 950g for 25 minutes at room temperature at the lowest acceleration setting and with the brake turned off. A cloudy buffy coat layer was then visible between the plasma and Ficoll, which consisted of PBMC. An illustration of the Ficoll gradient process is shown in Figure 3.1.

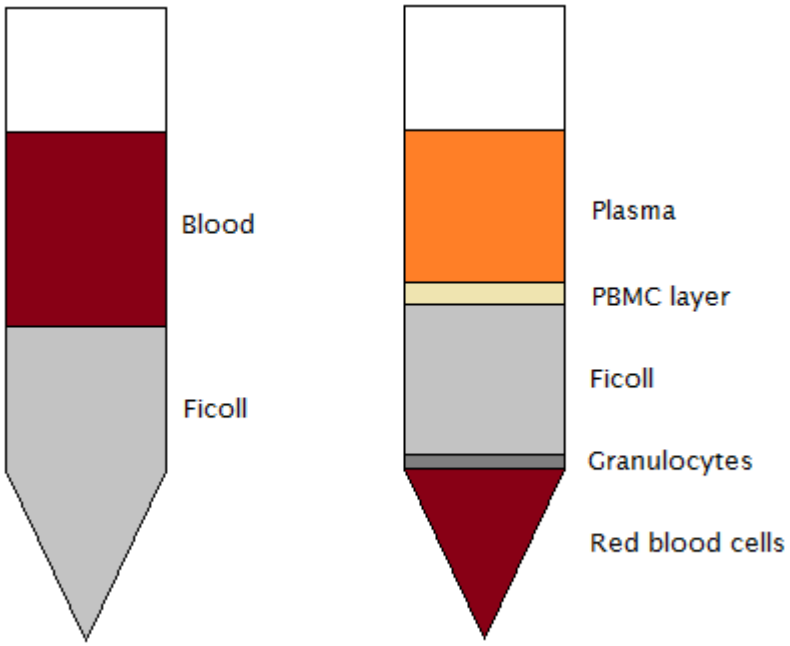


**Figure 3.1: Illustration of layers before (left) and after (right) centrifugation**

The PBMC layer was then removed using a Pasteur pipette and placed into a separate tube, which was topped up to 45ml with phosphate buffered saline (PBS) to act as a wash. The solution of PBMC and PBS was centrifuged at 700g for 8 minutes, resulting in a PBMC pellet at the bottom of the tube. The remaining PBS was poured off and the pellet then disturbed to loosen the cells. 1ml of PBS was then added and the mixture sampled by removing 10μl. This was added to 10μl of trypan blue, a stain taken up by dead cells, and a cell count performed using a haemocytometer and light microscope. The remaining PBMC/PBS solution was topped up with more PBS again to 45ml and washed for a second time by centrifugation. The remaining PBS was poured off and the cell pellet again disturbed to loosen the cells. Freezing medium (consisting of 80% heat-inactivated foetal calf serum and 20% dimethyl sulfoxide) was added to the PBMC pellet and disturbed to mix. PBMC samples were then separated into

1ml aliquots at a concentration of 1 million cells per ml. Samples were immediately placed in a chilled Thermo Scientific "Mr Frosty" container filled with propan-2-ol for slow freezing (-1ºC/minute) in a -80ºC freezer.

The PBMC samples were finally transferred in bulk and on dry ice to long-term liquid nitrogen storage at Southampton General Hospital within 4 weeks of the date of phlebotomy. Table 3.4 shows a list of reagents and equipment used in the PBMC isolation procedure.

**Table 3.4: Details of reagents and equipment used for isolation of PBMC**

| | Manufacturer | Product code |
|---|---|---|
| **Extractor hood** | LabGard Class II Biological Safety Cabinet from TripleRed | NU-437E-ES |
| **Centrifuge with swing buckets** | Eppendorf | 12863242 |
| **PBS (without Mg/Ca)** | Fisher Scientific | 12319922 |
| **Ficoll paque-plus** | Fisher Scientific | 11768538 |
| **Foetal calf serum** | Life tech | 10270-106 |
| **Trypan blue** | Sigma | T8154 |
| **Dimethyl sulfoxide (DMSO)** | Fisher Scientific | 10021310 |
| **"Mr Frosty" container** | Fisher Scientific | 10110051 |
| **Propan-2-ol** | Fisher Scientific | 10628143 |
| **Pipettes, centrifuge tubes (15ml and 50ml), cryovials, haemacytometer, light microscope** | Provided by Clinical Neurosciences, Faculty of Medicine, University of Southampton | |

### 3.1.7 Flow cytometry

Processing of PBMC using flow cytometry took place in the WISH laboratory at the University of Southampton. I received training from WISH staff Dr Yifang Gao and Dr Lindsey Chudley, who both supervised design of the antibody panel and initial experiments, with all actual experiments on patient samples

being performed by me. Experiments were performed in batch to reduce inter-experimental error. The flow cytometer, or fluorescence activated cell sorting (FACS) machine, used for PBMC analysis was the BD FACSCanto II system (BD Biosciences), configured with three lasers and eight colours. The software used to collect and analyse data was BD FACSDiva (BD Biosciences). Optimisation, configuration and maintenance of this equipment was performed by the WISH laboratory staff. Further general information about flow cytometry is presented below.

Flow cytometry is routinely used in the diagnosis and research of medical disorders, especially in the field of cancer immunology. The flow cytometer uses fluidics to allow a suspension of cells to pass through optical lasers, which are used as excitation sources that cause the scatter of light when they hit each cell. The level of forward scattered light can be measured and depends on the size of the cell. Side scatter can also be measured and indicates the level of granularity within the cell. A combination of forward scatter and side scatter signals can help to differentiate types of PBMC. For example, lymphocytes are generally small in size and contain minimal granules. In contrast, granulocytes (e.g. eosinophils) are generally large and have a more granular cytoplasm. Flow cytometers can also be used to separate and isolate cell populations to allow culture and investigation of individual PBMC subsets.

Signals from dead and doublet cells can be removed from analysis through careful gating of populations. Fluorochrome labelled antibodies are used to pre-label cells and these emit light of a specific wavelength when excited by the optical laser. The wavelength of light is then detected and recorded, giving data on the excitation profile of each individual cell. A database is therefore built of the number and type of cells marked with a variety of different fluorochromes. This gives information regarding the type and number of cells in a sample. Figure 3.2 illustrates this using a schematic of a flow cytometer. The figure illustrates cell samples that are taken up by the flow cytometer nozzle and pushed through a laser in single file. Individual lasers are aimed at the cell stream and scattered light collected by multiple readers. Cells tagged with particular antibody fluorochromes emit light of a specific wavelength, which is information that is detected and processed by an analysis workstation.

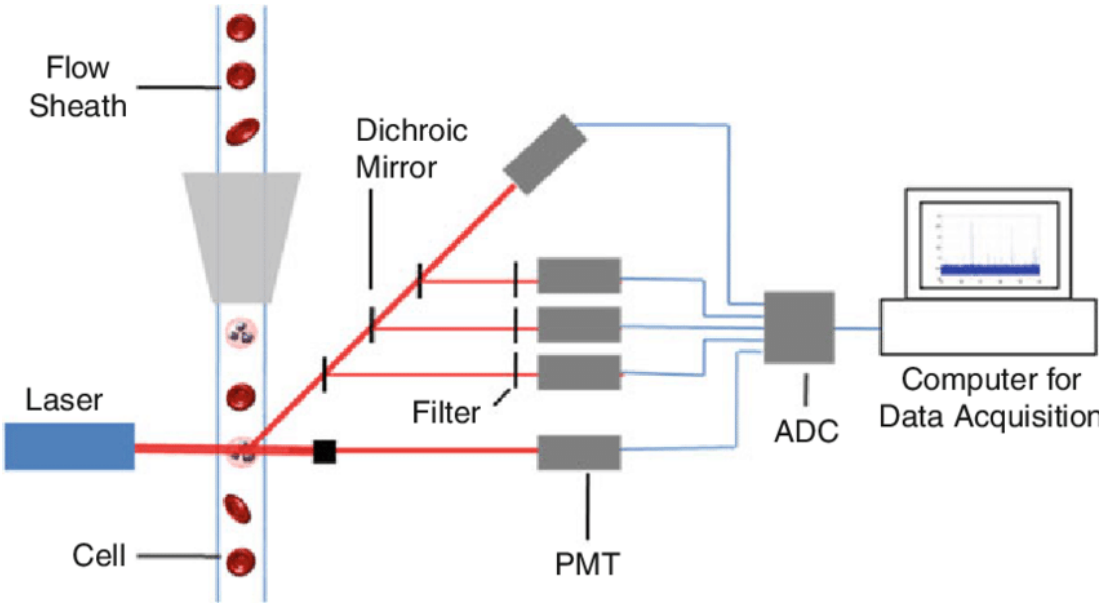


**Figure 3.2: Schematic of flow cytometer**
Schematic diagram of a flow cytometer. Image taken from Wei et al. [370]. PMT=photomultiplier tube. ADC=analog-to-digital converter.

Table 3.5 shows a list of reagents used in the flow cytometry experiments. All reagents were prepared and stored as per the local WISH laboratory standard operating procedures.

**Table 3.5: Details of reagents used in flow cytometry**

| Reagent | Method of preparation |
|---|---|
| **Complete medium** | 90% RPMI (Roswell Park Memorial Institute) medium, minus glutamate<br>10% heat inactivated foetal calf serum, Gibco<br>Mixed and stored at 4ºC |
| **FACS buffer** | 500ml phosphate buffered saline (PBS)<br>2.5g bovine serum albumin (BSA)<br>500µl sodium azide<br>Mixed and stored at 4ºC |
| **Zombie cell viability dye** | Viability kit contained lyophilised Zombie Violet dye and anhydrous DMSO. 100µl of DMSO added to Zombie and mixed. Stored in aliquots at -20ºC. |

Details of the antibody fluorochrome panel used to label PBMC subsets during flow cytometry experiments are listed in table 3.6, with further details of each antibody listed below.

- Zombie is a marker that is taken up by dead cells and is therefore used to exclude dead cells from analysis
- CD3 is a pan-T cell marker
- CD4 and CD8 are markers of helper T cells and cytotoxic T cells respectively
- CD45RA is a marker of naïve T cells, the absence of which indicates a memory phenotype
- CCR7, C-C chemokine receptor type 7, is expressed on T cells that localise towards central lymphoid tissue, the absence of which indicates an effector phenotype
- CD14 is a marker of monocytes
- CD19 is a B cell marker
- HLA-DR is a non-transient activation marker of PBMC that indicates an antigen presentation activated phenotype

These markers allow for the definition of PBMC subsets, based on the combination of their expression on the cell surface. The co-expression of CD45RA and CCR7 is particularly complex, and allows for the recognition of 4 subsets of helper T cells and cytotoxic T cells: naïve T cells are CCR7+CD45RA+, central memory T cells are CCR7+CD45RA-, effector memory T cells are CCR7-CD45RA- and "terminally differentiated effector memory cells re-expressing CD45RA" (TEMRA) are CD45RA+CCR7- [195, 371, 372].

Naïve T cells essentially have not encountered any antigen to date. Central memory T cells focus on proliferation in response to antigen encounter, while effector memory T cells localise to peripheral tissue. It is noteworthy that the expression of CD45RA is usually associated with naïve T cells. However, a subset of effector memory T cells re-express CD45RA after stimulation with antigen, and are referred to as TEMRA. These cells are associated with chronic immune activation, for example as a result of viral or bacterial infection [195, 372]. Further details of cell populations identified by combinations of the markers listed above are presented in table 3.7.

**Table 3.6: Details of antibody fluorochrome panel**

| Marker | Fluorochrome | Manufacturer | Product code | Clone | Volume per case | Cell population detected |
|---|---|---|---|---|---|---|
| **Zombie** | Pacific Blue | BioLegend | 423113 | Violet | 1μl | Dead cells |
| **CD3** | PerCP | Becton-Dickinson | 345766 | SK7 | 20μl | T cell (pan) |
| **CD4** | V500 (AmCyan) | BD Biosciences | 560768 | RPAT4 | 5μl | T cell (helper) |
| **CD8** | PE | Fisher | 12-0087-42 | SK1 | 5μl | T cell (cytotoxic) |
| **CD14** | PerCP-Cy5.5 | Fisher | 45-0149-42 | 61D3 | 5μl | Monocyte |
| **CD19** | FITC | Fisher | 11-0199-42 | HIB19 | 5μl | B cells |
| **CD45RA** | APC | Fisher | 17-0458-42 | HI100 | 5μl | T cells (naive) |
| **CCR7** | APC-Cy7 | Biolegend | 353212 | G043H7 | 5μl | T cells (central) |
| **HLA-DR** | PE-Cy7 | Fisher | 25-9956-42 | LN3 | 5μl | Activation (antigen presentation) |

**Table 3.7: List of markers corresponding to PBMC subsets**

| Cell population | Corresponding markers |
|---|---|
| **T cell** | CD3+ |
| **Helper T cell** | CD3+ CD4+ |
| **Activated helper T cell** | CD3+ CD4+ HLA-DR+ |
| **Cytotoxic T cell** | CD3+ CD8+ |
| **Activated cytotoxic T cell** | CD3+ CD8+ HLA-DR+ |
| **Double positive T cells** | CD3+ CD4+ CD8+ |
| **Double negative T cells** | CD3+ CD4- CD8- |
| **Naïve T cell** | CD3+ CD4/8+ CCR7+ CD45RA+ |
| **Central memory T cell** | CD3+ CD4/8+ CCR7+ CD45RA- |
| **Effector memory T cell** | CD3+ CD4/8+ CCR7- CD45RA- |
| **TEMRA** | CD3+ CD4/8+ CCR7- CD45RA+ |
| **B cells** | CD3- CD19+ |
| **Activated B cells** | CD3- CD19+ HLA-DR+ |
| **Monocytes** | CD3- CD14+ |
| **Activated monocytes** | CD3- CD14+ HLA-DR+ |

CD = Cluster of differentiation
TEMRA = terminally differentiated effector memory cells re-expressing CD45RA

Experiments to optimise the flow cytometer and antibody panel were conducted under the supervision of WISH laboratory staff. The flow cytometer was periodically calibrated by myself. 9 samples were prepared using WISH stock control PBMC – one for each of the 8 cytometer fluorochromes plus one that was unstained. Each sample was tested in the flow cytometer with parameters adjusted to ensure adequate compensation and minimisation of spill-over of antibody fluorochrome to adjacent light wavelengths. The BD FACSDiva software was then used to automatically compensate for any spill-over, with settings then applied to all remaining samples.

In addition, flow cytometry control experiments were run before any samples were processed. Fluorescence-minus-one (FMO) control experiments were performed in order to allow accurate interpretation of flow cytometry data. FMO control experiments included all fluorochromes in the panel, minus one of them, and can also be described as negative control experiments. They

allow a boundary to be set for what constitutes positive or negative staining for each marker, thus ensuring that the cell population identified in experiments is truly positive for each specific marker. A list of each FMO panel used follows.

- ALL (Zombie, CD3, CD4, CD8, CD14, CD19, CD45RA, CCR7, HLA-DR)
- No Zombie (CD3, CD4, CD8, CD14, CD19, CD45RA, CCR7, HLA-DR)
- No CD3 (Zombie, CD4, CD8, CD14, CD19, CD45RA, CCR7, HLA-DR)
- No CD4 (Zombie, CD3, CD8, CD14, CD19, CD45RA, CCR7, HLA-DR)
- No CD8 (Zombie, CD3, CD4, CD14, CD19, CD45RA, CCR7, HLA-DR)
- No CD14 (Zombie, CD3, CD4, CD8, CD19, CD45RA, CCR7, HLA-DR)
- No CD19 (Zombie, CD3, CD4, CD8, CD14, CD45RA, CCR7, HLA-DR)
- No CD45RA (Zombie, CD3, CD4, CD8, CD14, CD19, CCR7, HLA-DR)
- No CCR7 (Zombie, CD3, CD4, CD8, CD14, CD19, CD45RA, HLA-DR)
- No HLA-DR (Zombie, CD3, CD4, CD8, CD14, CD19, CD45RA, CCR7)

The final protocol used for analysis of PBMC samples follows. Cryopreserved PBMC samples were removed from liquid nitrogen on dry ice and quick-thawed using a water bath pre-warmed to 37ºC. PBMC samples were then washed by being mixed with warm complete medium and centrifuged at 300g for 5 minutes. Supernatant was poured off and the cell pellet then flicked to be re-suspended. This wash was then repeated once more. 1μl of Zombie violet cell viability dye and 100μl of PBS was added to the cell sample and incubated in the dark at room temperature for 15 minutes. In that time a master-mix of antibody fluorochromes was prepared on ice. All manufacturer recommended volumes of antibody were added to one vial and mixed. Following completion of incubation with Zombie, the cell samples were washed in FACS buffer and spun at 1500rpm for 5 minutes. 100μl of FACS buffer was added to each sample and an equal amount of the antibody master-mix was also added, before incubation in an ice box (with the lid closed) for 30 minutes. Samples were washed twice as above, with the supernatant discarded each time. Lastly, 150μl of FACS buffer was added to each sample and all samples kept on ice until processing, generally for a maximum of 30 minutes.

Each PBMC sample was run on the flow cytometer in turn and data captured in real time. Typically each sample was run until data was collected on at least

10,000 T cells in order to ensure sufficient data for analysis. Data for each sample was recorded and saved separately.

A gating procedure was agreed upon to allow visualisation and quantification of PBMC subsets. An example of the gating strategy used in SILAD is shown in figure 3.3. The list below describes the order through which specific cell populations were gated and identified from the parent population.

1. All cell data-points were initially plotted on a histogram as forward scatter height vs forward scatter, allowing for visualisation and exclusion of any doublet cells.
2. Gated singlet cells were then plotted on Zombie vs forward scatter, allowing for gating on live singlets.
3. Live singlets were then plotted on side scatter vs forward scatter. This allowed visualisation of a lymphocyte population and a larger (by size) monocyte population.
4. A gate was drawn around the monocyte population which was then plotted on side scatter vs CD14, allowing for measurement of CD14+ monocytes.
5. Lymphocytes were gated and plotted as CD19 vs CD3, allowing for a population of CD3+CD19- cells to be selected – the T cell population.
6. Concurrently a CD3-CD19+ population was visible – the B cell population.
7. CD3+ T cells were then plotted as CD8 vs CD4. This allowed four populations to be revealed: CD3+ T cells that were a) CD4-CD8-, b) CD4+CD8+, c) CD4-CD8+ and d) CD4+CD8-. The CD4+CD8- cells were deemed to be helper T cells whilst the CD4-CD8+ cells deemed to be cytotoxic T cells.
8. Each of these two populations were separately plotted on CCR7 vs CD45RA. This allowed a further four populations to be revealed for both helper and cytotoxic T cells: a) CCR7+CD45RA+, b) CCR7+CD45RA-, c) CCR7-CD45RA- and d) CCR7-CD45RA+. This provided data on the proportion of T cells that were either a) naïve, b) central memory, c) effector memory, or d) TEMRA.
9. Lastly, CD4+ T helper cells, CD8+ T cytotoxic cells, CD19+ B cells and CD14+ monocytes were all separately plotted against HLA-DR. Gates were set to measure the activated percentage of all of these different cell types.

PBMC subset population data was recorded as the percentage of parent cell population. For example, the CD4+ cell population was recorded as the

percentage of CD3+ cells that were CD4+. The reason for using this method, rather than absolute cell counts, was two-fold. Firstly, there may have been differences in the number of cells retrieved and processed for each sample, which would have directly affected absolute counts. Secondly, the gating strategy used is based on visual determination of cell populations based on their distributions on scatter graphs, meaning that a slight shift of gate could lead to a change in subset population. In order to reduce any bias caused by these two factors, it was decided that percentage of parent cell population should be used. This method of reporting PBMC subset data is widely used.

One cell population was measured using an alternative method. HLA-DR positive cell populations (on helper T cells, cytotoxic T cells, B cells and monocytes) were gated on HLA-DR+ populations. The positive population was then analysed for “mean fluorescent intensity”. This outcome measures the mean intensity of the fluorescent signal from the HLA-DR positive cell population. This measure is widely used in flow cytometry to quantify the extent of activation of cell subsets, rather than using cell population proportions.

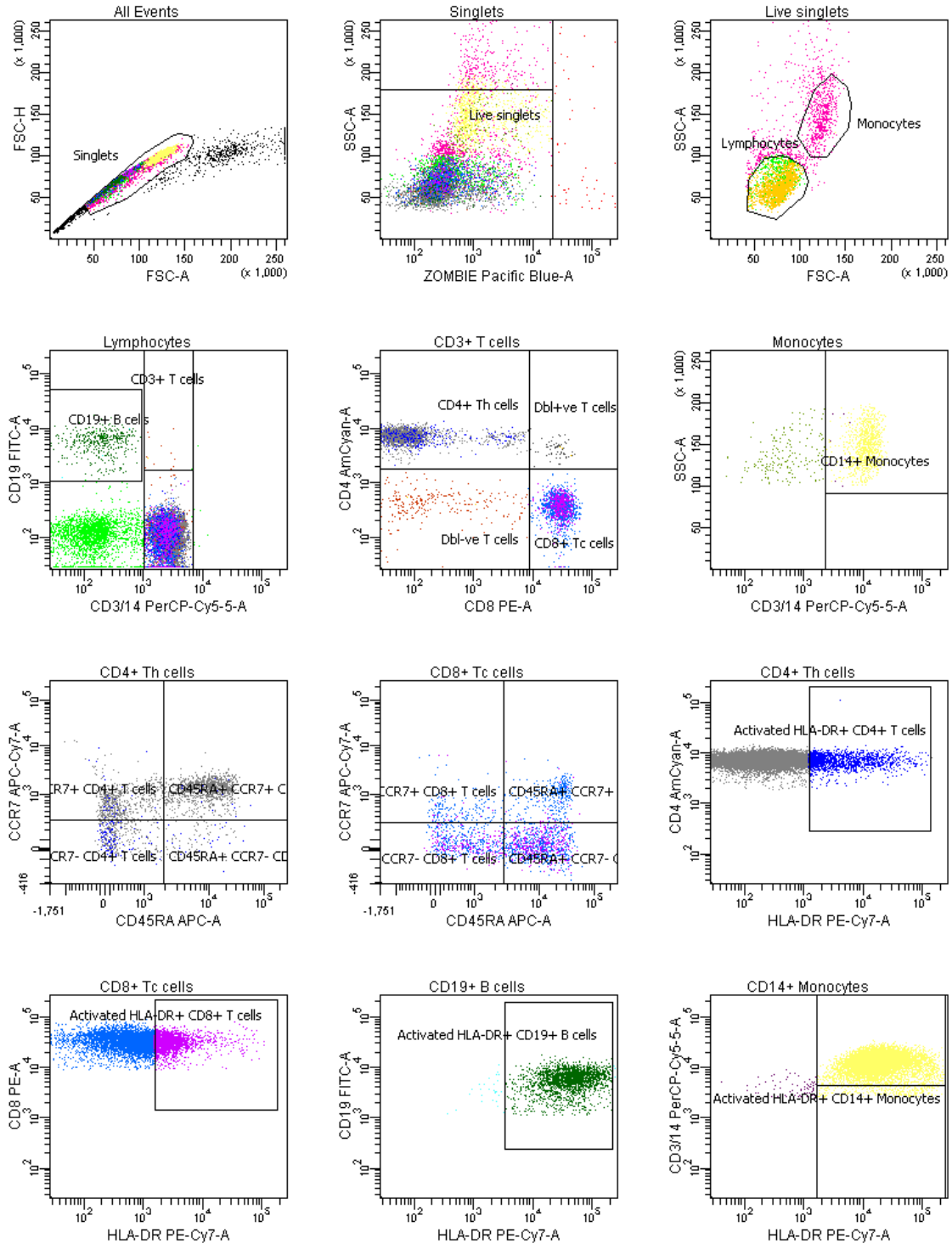


**Figure 3.3: Example of flow cytometry gating strategy**
Series of histograms showing immunolabelled cells to allow identification of PBMC subsets. Live singlets are identified on the top row. T cells and monocytes are then identified in the second row, followed by examination of the different T cell subsets in the third row. Lastly, the proportion of activated PBMC are examined in the bottom row.

### 3.1.8 Stimulation of PBMC

In addition to phenotypic analysis of PBMC sub-populations, PBMC were also stimulated *ex-vivo* with LPS and PHA, followed by collection of supernatant for cytokine analysis.

Advice was provided by WISH laboratory staff regarding the concentration of agents to be used to stimulate PBMC, with the aim of producing optimal stimulation of cytokine production. Concentrations used to stimulate PBMC in previous relevant studies ranged from 1ng/ml [356] to 10μg/ml [373] for LPS, and from 2.5μg/ml [245] to 20μg/ml [251] for PHA. Stimuli were prepared in the following manner. 10ml of sterile PBS was added to 1mg of LPS (Sigma, L4391), to obtain a concentration of 1μg/10μl, split into aliquots that were stored at -20ºc. LPS solution was used at 1:100, giving a final concentration of 1μg/1ml. In addition, 10ml of sterile PBS was added to 5mg of PHA (Sigma, L1668), to obtain a concentration of 5μg/10μl, split into aliquots stored at -20ºc. PHA solution was used at 1:100, giving a final concentration of 5μg/1ml.

Similar to methods described at the start of this section, PBMC samples were quick-thawed from liquid nitrogen and then washed with warm complete medium. The solution of cells was centrifuged at 300g for 5 minutes before the supernatant was poured off. 1ml of complete medium was then added to each sample before it was mixed and split equally into three tubes. One tube was subjected to the addition of 1:100 of LPS stock solution, another with 1:100 of PHA stock solution and the third left unstimulated. Each set of three tubes per sample were incubated for 16 hours in a tissue culture warmer (37ºC). Following incubation the cell solutions were spun at 1500rpm for 5 minutes and the supernatant carefully removed so as not to disturb the cell pellet. Supernatant was then stored at -20ºC until batch analysis.

### 3.1.9 Multiplex immunoassay

Multiplex analysis was used to assess the concentrations of cytokines in serum and in stimulated PBMC supernatant. Meso Scale Discovery (MSD) was the platform chosen in this study. The “MSD V-PLEX pro-inflammatory panel 1 human kit” (Meso Scale Diagnostics LLC, K15049D-2) was selected as it measured a range of cytokines known to be important in inflammation and

immune system regulation. The panel of ten cytokines consisted of: IL1β, IL2, IL4, IL6, IL8, IL10, IL12, IL13, TNFα and IFNγ. Further details regarding each cytokine are listed below.

- IL1β is a key pro-inflammatory and pyrogenic cytokine
- IL2 is also known as T-cell growth factor and is a pro-inflammatory cytokine and regulator of T-cell proliferation
- IL4 is an anti-inflammatory cytokine produced by alternatively activated cells to activate B cells
- IL6 is a pro-inflammatory cytokine secreted by T cells and macrophages, which also has anti-inflammatory functions.
- IL8 is a pro-inflammatory cytokine involved in attracting T cells and in neutrophil activation.
- IL10 is an anti-inflammatory cytokine produced by T cells and macrophages, which functions to inhibit the synthesis of a range of pro-inflammatory cytokines
- IL12 is a pro-inflammatory cytokine produced by T cells and macrophages to activate T cells and natural killer cells
- IL13 is an anti-inflammatory cytokine involved in regulation of B cell proliferation and macrophage activation
- TNFα is a key pro-inflammatory cytokine that stimulates IL1 and can induce inflammation
- IFNγ is a pro-inflammatory cytokine produced by lymphocytes and activates macrophages.

The use of multiplex plates to measure cytokine levels in serum and stimulated PBMC supernatant was performed by Dr Laurie Lau (University of Southampton), aided by myself. The V-PLEX multi-spot assay plate used in this study can be described as a sandwich immunoassay. These types of plates typically arrive pre-coated with antibodies against a panel of cytokines, located in well-defined spots at the bottom of 96 individual wells. Serum or supernatant samples are then added to each well with a solution containing detection antibodies conjugated with electrochemiluminescent labels. An MSD buffer is added to the solution which allows the plate reading machine (MESO QuickPlex SQ 120 imager) to pass a voltage through and trigger the electrochemiluminescent labels to emit light, which is then quantified.

During the preparation for these experiments, calibrator dilutions were formulated using the MSD calibrator and diluent provided in the plate kit. Serum or supernatant samples were defrosted and then centrifuged for 3 minutes at 2000g to remove particulates. Aliquots of serum or supernatant were used to avoid repeated freeze-thaw cycles. Samples were then diluted using kit diluent. The antibody detection solution, wash buffer and Read-buffers were prepared using manufacturer's instructions.

At the start of the assay the multiplex plate was washed 3 times with wash buffer. Diluted calibrators were added to a proportion of wells, as per manufacturer protocol, with remaining wells filled with serum or supernatant samples. Serum samples were used at 1:2 dilution, unstimulated supernatant was used neat, while stimulated supernatant was used at either 1:5 or 1:10 dilution. An adhesive plate seal was applied and the plate incubated at room temperature for 2 hours on a microplate shaker. Following this, the plate was washed 3 times with wash buffer before addition of the Read-buffer to each well. The plates were immediately loaded onto the Quickplex SQ 120 imager, loaded with Discovery Workbench software. This allowed quantification of individual cytokine concentrations for each sample. Batch analysis was performed to minimise inter-experimental variation in results.

All serum and supernatant samples were processed in duplicate and the mean value taken per cytokine per case. Any cytokine concentration below the lower limit of detection (LLOD), as specified by the manufacturer, was deemed to be 0. If the cytokine concentration in one of the two duplicate samples was below the LLOD or not detectable, then the single valid concentration was used. A list of LLODs for each cytokine used in this panel is shown in table 3.8. It should be noted that data for IL8 concentrations appeared unreliable and mostly lower than the level of detection so were repeated in one batch.

**Table 3.8: Lower limits of detection for cytokines in multiplex assay**

| Cytokine | Lower limit of detection (pg/ml) |
|---|---|
| **IL1β** | 0.04 |
| **IL2** | 0.09 |
| **IL4** | 0.02 |
| **IL6** | 0.06 |
| **IL8** | 0.04 |
| **IL10** | 0.03 |
| **IL12** | 0.11 |
| **IL13** | 0.24 |
| **TNFα** | 0.04 |
| **IFNγ** | 0.20 |

### 3.1.10 Quality assurance

Potential participants to SILAD were identified through a) referrals from local clinicians, b) the website Join Dementia Research, or c) self-referrals. Discrepancies in patient or carer-reported past medical history, drug history or diagnosis were checked with referral information or checked with the participant's GP to ensure accuracy.

Initiation visits were made prior to the additional study site (Solent NHS Trust) starting recruitment, for the purpose of training on study procedures and rating scales. Staff from the Solent site then shadowed several study visits at the MARC site for further training on consent, study procedures and rating scales. Following commencement of recruitment, several visits were made to the Solent site to ensure consistency of data collection across both sites. Data collection was checked at one time point midway through the recruitment period of Solent, and at the end of recruitment, to ensure quality of data and adherence to study protocols. Discrepancies with regards to typographical errors on the consent form and scoring of two of the rating scales were identified and rectified at the midway visit.

Data entry into an electronic database was performed by an undergraduate medical student, under my supervision. I then sampled every 9th case (in recruitment order) and double-checked all data entry points for those cases. No incorrect data had been recorded. However, several points of data were missing with regards to smoking history, family history and time of phlebotomy. I then checked the entire SPSS spreadsheet for missing data and completed the gaps by referring to source documentation. The collection of information regarding duration of disease was performed retrospectively, following the completion of the study, using information from patient records where available.

### 3.1.11 Data analysis

Study data was entered into statistical software SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). This included the date and times of each visit, demographic details, diagnostic history, medical history, drug history, neuropsychological test scores and all blood analysis data. Graphs were prepared using GraphPad Prism (Version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

All continuous variables were assessed for normality using histograms and QQ plots. Parametric or non-parametric tests were used depending on the distribution of each variable.

Baseline comparisons were made to check for significant differences between the three groups (DLB, AD and controls) for age, gender, years of formal education and *APOE* genotype, using ANOVA or Chi-squared tests, depending on the type of variable. Significant group differences in the proportion of participants who were positive for core DLB clinical features, positive for recent inflammatory events and positive for chronic risk factors and drug treatments were all assessed using parametric or non-parametric tests.

Serum cytokine values were recorded as pg/ml, but were reclassified as 0 if found to be below the lower limit of detection for the assay. Lymphocyte cell populations were recorded as percentages of the parent population rather than absolute numbers, to avoid errors associated with inter-case differences in absolute cell numbers. HLA-DR positive cell populations were measured as

mean fluorescent intensity. Supernatant cytokine data from stimulated PBMC was recorded as a ratio of stimulated cytokine concentration divided by unstimulated cytokine concentration, again to avoid errors associated with inter-case differences in absolute cell numbers.

Statistical tests were then used to examine for significant group differences in cytokine concentrations, lymphocyte cell populations and stimulated cytokine concentrations. Significance was determined if two-tailed $P<0.05$ for group differences, with post-hoc adjustment where appropriate. Correlations were performed to check for statistically significant associations between inflammatory markers and the clinical features of DLB. Two-tailed $P<0.01$ was deemed to be significant to allow for testing of multiple correlations.

## 3.2 Results

95 participants were recruited to SILAD; 32 with a diagnosis of probable DLB, 31 with a diagnosis of probable AD and 32 cognitively intact controls. One patient was recruited to the AD group and subsequently excluded due to the diagnosis of dementia being withdrawn by the Solent site principle investigator. Furthermore, it should be noted that phlebotomy failed on two participants (one control and one DLB) but their clinical data were still included.

### 3.2.1 Baseline characteristics

Assessment of the baseline characteristics of participants was performed. Table 3.9 shows a summary of baseline data for age, gender, number of years of formal education, disease duration and *APOE* genotype.

**Table 3.9: Baseline characteristics for SILAD**

| | Controls | DLB | AD |
|---|---|---|---|
| Age (years±SD) | 66.1±6.6 | 73.9±7.5 | 74.1±7.4 |
| Gender (M:F) | 15:17 | 20:12 | 18:13 |
| Years of education (years±SD) | 12.8±2.5 | 12.7±3.1 | 13.3±3.8 |
| Disease duration (years±SD) | - | 3.8±1.7 | 4.5±2.4 |
| *APOE* genotype ≥1 ε4 allele, (%) | 7 (22.6%) | 15 (48.4%) | 20 (64.5%) |

Continuous variables presented as mean ± standard deviation

The age of participants was deemed to be normally distributed following analysis of QQ plots and Shapiro-Wilk tests. There was a statistically significant difference between the three groups as determined by one-way ANOVA ($(F2,92)=10.816$, $P<0.001$). A Tukey post hoc test revealed that the age of participants was statistically significantly higher in the AD (74.10±7.38 years, $P<0.001$) and DLB (73.91±7.49 years, $P<0.001$) groups compared to controls (66.09±8.56 years). There was no statistically significant difference in age between AD and DLB ($P=0.995$).

The DLB and AD groups comprised more males than females, whilst the control group included more females than males. Overall, there was no significant difference in gender between the three groups as determined by Pearson Chi-squared test ($\chi^2(2)=1.68$, P=0.432).

The distribution of years of formal education in each of the three groups was plotted on QQ plots to test for normality. Control and AD groups were shown to be non-normally distributed, while the DLB group was shown to be normally distributed. There was no statistically significant difference between the three groups as determined by Kruskal-Wallis test (KW=0.229, P=0.892).

The distribution of duration of disease was plotted on QQ plots to test for normality. The DLB group was shown to be normally distributed whilst the AD group was not. There was no significant difference in duration of disease between the DLB group (mean rank 22.28) and the AD group (mean rank 26.32) as determined by Mann-Whitney U test (MWU=316.5, P=0.309).

The majority of control subjects did not possess an *APO* ε4 allele, whereas approximately two thirds of AD subjects and one half of DLB subjects did possess at least one *APO* ε4 allele. There was a significant difference in possession of at least one *APO* ε4 allele between the three groups, as determined by Pearson Chi-squared test ($\chi^2(2)=11.202$, P=0.004).

### 3.2.2 Diagnostic criteria for DLB

All participants were assessed for core and suggestive features of DLB. Table 3.10 shows that the majority of DLB patients had each of the three core features of DLB (using the third international consensus criteria [9]), along with REM sleep behaviour disorder and reduced DaT on PET/SPECT imaging. The differences between groups for the core features of DLB were all significant (see table for significance values). Neuroleptic sensitivity did not show a significant difference between groups.

**Table 3.10: Diagnostic criteria for DLB in SILAD**

| | Controls | DLB | AD | P |
|---|---|---|---|---|
| Fluctuating cognition | 0 | 28 (87.5%) | 0 | **70.06 (P<0.001)** |
| Visual hallucinations | 0 | 23 (71.9%) | 0 | **52.982 (P<0.001)** |
| Parkinsonism | 0 | 28 (87.5%) | 0 | **70.06 (P<0.001)** |
| REM sleep behaviour disorder | 0 | 22 (68.8%) | 2 (8.7%) | **42.752 (P<0.001)** |
| Neuroleptic sensitivity | 0 | 2 (6.3%) | 0 | 3.455 (P=0.178) |
| Reduced DaT on PET/SPECT | 0 | 17 (54.8%) | 0 | **37.016 (P<0.001)** |

Results are presented as number positive for criterion (percentage of total)
P is Pearson Chi-squared statistic (P value)

### 3.2.3 Inflammatory events

Binary data was recorded as to whether participants experienced specific inflammatory events during the six weeks prior to the study visit.

Table 3.11 shows a summary of the number of inflammatory events in each group. There was no statistically significant difference in the frequency of infections, episodes of surgery or frequency of vaccinations between groups. There was a statistically significant difference in the frequency of trauma between the three groups, with the DLB group having the highest number of participants experiencing physical or psychological trauma in the preceding six weeks.

**Table 3.11: Inflammatory events in SILAD**

| | Controls | DLB | AD | P |
|---|---|---|---|---|
| Infections | 4 (12.5%) | 11 (34.4%) | 7 (22.6%) | 4.311 (P=0.116) |
| Surgery | 0 | 2 (6.3%) | 2 (6.5%) | 2.057 (P=0.357) |
| Vaccinations | 2 (6.3%) | 3 (9.4%) | 5 (16.1%) | 2.057 (P=0.357) |
| Trauma | 4 (12.5%) | 12 (37.5%) | 2 (6.5%) | **11.190 (P=0.004)** |

Results are presented as number positive for criterion (percentage of total)
P is Pearson Chi-squared statistic (P value)

### 3.2.4 Chronic risk factors

All participants were assessed for their past medical history, including whether they had hypertension, cerebrovascular disease, rheumatoid arthritis, ischaemic heart disease, diabetes mellitus and hypercholesterolaemia.

Table 3.12 shows the numbers of participants in each group with each of the above chronic risk factors, along with the Pearson Chi-squared test value and P value of significance. There were no statistically significant differences between groups in the number of participants who had any of the chronic risk factors listed above.

**Table 3.12: Chronic risk factors in SILAD**

| | Controls | DLB | AD | P |
|---|---|---|---|---|
| Hypertension | 14 (43.8%) | 15 (46.9%) | 11 (35.5%) | 0.892 (P=0.640) |
| Cerebrovascular disease | 2 (6.3%) | 7 (21.9%) | 2 (6.5%) | 4.997 (P=0.082) |
| Rheumatoid arthritis | 3 (9.4%) | 3 (9.7%) | 2 (6.5%) | 0.254 (P=0.881) |
| Ischaemic heart disease | 3 (9.4%) | 8 (25.0%) | 3 (9.7%) | 4.046 (P=0.132) |
| Diabetes mellitus | 4 (12.5%) | 1 (3.1%) | 6 (19.4%) | 4.091 (P=0.129) |
| Hypercholesterolaemia | 6 (18.8%) | 12 (37.5%) | 14 (45.2%) | 5.232 (P=0.073) |

Results are presented as number positive for criterion (percentage of total)
P is Pearson Chi-squared statistic (P value)

### 3.2.5 Drug treatments

Participants were assessed for their drug history, including whether they were taking cognitive enhancer medication (cholinesterase inhibitors or Memantine), medicines for Parkinsonism or anti-inflammatory drugs. Table 3.13 shows the numbers of participants in each group taking these drug treatments.

No control participants were taking any memory enhancer medication or medicines for Parkinsonism. Significantly more DLB and AD patients were taking memory enhancer medications than control subjects. Significantly more DLB patients were taking antipsychotic medications and medicines for Parkinsonism than the control and AD groups. There was no statistically significant difference in use of NSAIDs or oral steroids between groups.

**Table 3.13: Drug treatments in SILAD**

| | Controls | DLB | AD | P |
|---|---|---|---|---|
| Cholinesterase inhibitors | 0 | 30 (93.8%) | 24 (77.4%) | **65.266 (P<0.001)** |
| Memantine hydrochloride | 0 | 3 (9.4%) | 4 (12.9%) | 4.126 (P=0.127) |
| Antipsychotic medications | 1 (3.1%) | 7 (21.9%) | 1 (3.2%) | **8.653 (P=0.013)** |
| Medications for Parkinsonism | 0 | 8 (25%) | 0 | **17.198 (P<0.001)** |
| Non-steroidal anti-inflammatory drugs | 7 (21.9%) | 7 (21.9%) | 8 (25.8%) | 0.181 (P=0.913) |
| Oral steroids | 0 | 2 (6.3%) | 1 (3.2%) | 2.044 (P=0.360) |

Results are presented as number positive for criterion (percentage of total)
P is Pearson Chi-squared statistic (P value)

### 3.2.6 MoCA

The distribution of all cognitive and neuropsychiatric test scores were tested for normality using QQ plots. All test scores were deemed to be non-normal in distribution.

Group differences in MoCA score were assessed using the Kruskal-Wallis test, with post-hoc analysis using Dunn-Bonferroni to test for significant pairwise differences. Table 3.14 shows a summary of MoCA scores in each group. There was a statistically significant difference in MoCA score between the three groups as determined by the Kruskal-Wallis test (Kruskal-Wallis=62.208, P<0.001), with a mean rank MoCA score of 79.17 for Controls, 33.63 for AD and 30.75 for DLB.

Post-hoc pairwise comparisons using Dunn-Bonferroni tests showed statistically significant lower MoCA score in DLB compared to controls (P<0.001), and lower MoCA score in AD compared to controls (P<0.001). There was no significant difference between AD and DLB (P=1.000).

**Table 3.14: MoCA scores in SILAD**

| | Controls | DLB | AD | P |
|---|---|---|---|---|
| MoCA | 29±2 | 19±8 | 20±7 | **62.208 (P<0.001)** |

Results are presented as median ± inter-quartile range
P is Kruskal-Wallis test statistic (P value)

### 3.2.7 Neuropsychiatric tests

Mann-Whitney U tests were used to check for statistically significant differences between DLB and AD groups for all other neuropsychiatric tests, as there was no third group to compare with. Table 3.15 shows a summary of neuropsychiatric test scores in SILAD, with findings summarised below.

There was no significant difference in either FCSRT-IR total score (Mann-Whitney U=362.5, P=0.194) or free recall score (Mann-Whitney U=380.5, P=0.307) between DLB and AD groups.

There was no significant difference in CSDD score (Mann-Whitney U=491.5, P=0.950) between DLB and AD groups.

CAF score was significantly higher in the DLB group compared to the AD group (Mann-Whitney U=118.5, P<0.001).

The UPDRS score, and all subsection within, was significantly higher in the DLB group compared to the AD group (Mann-Whitney U=71, P<0.001 for total score).

The total NPI score was significantly higher in the DLB group compared to the AD group (Mann-Whitney U=346.5, P=0.04). However, the total NPI carer distress score was not significantly different between the two groups (Mann-Whitney U=362.5, P=0.065).

The NPI scores of each domain were also tested for differences between the two dementia groups. DLB participants scored significantly higher than the AD group on the following domains: delusions (Mann-Whitney U=348.0, P=0.005), hallucinations (Mann-Whitney U=163.0, P<0.001) and apathy (Mann-Whitney U=283.5, P=0.003). The AD group scored higher than the DLB group on irritability/lability (Mann-Whitney U=648.0, P=0.018). There were no significant group differences for agitation/aggression, depression, anxiety, elation, disinhibition and motor behaviour.

**Table 3.15: Neuropsychiatric test scores in SILAD**

| | DLB | AD | P |
|---|---|---|---|
| FCSRT-IR sum total | 45 (11) | 42 (28) | 362.5 (P=0.194) |
| FCSRT-IR free recall | 18 (16) | 14 (16) | 380 (P=0.307) |
| CSDD | 2 (3) | 2 (3) | 491.5 (P=0.950) |
| CAF | 4 (6) | 0 (0) | **118.5 (P<0.001)** |
| UPDRS total | 32 (26) | 7 (7) | **71 (P<0.001)** |
| UPDRS motor examination | 14.5 (13) | 2 (5) | **107.5 (P<0.001)** |
| NPI total | 9.5 (14) | 6 (8) | **365.5 (P=0.040)** |
| NPI carer distress | 6.5 (10) | 4 (5) | 363.5 (P=0.065) |

Results are presented as median (IQR)
P is Mann-Whitney U test statistic (P value)
FCSRT-IR = Free and cued selective reminding test – immediate recall
CSDD = Cornell scale for depression in dementia
CAF = Clinician assessment of fluctuation
UPDRS = Unified Parkinson's disease rating scale
NPI = Neuropsychiatric inventory

### 3.2.8 Flow cytometry

The distribution of PBMC populations were tested for normality by QQ plots, with table 3.16 listing the distribution types of these variables. Non-normally distributed variables were transformed using Lg10 and then re-tested for normality. In most cases the Lg10 transformed data remained non-normal. ANOVA was therefore used to assess for differences between groups for normally distributed variables, with Tukey post-hoc tests used to assess for paired group differences. The Kruskal-Wallis test was used for non-normal variables.

**Table 3.16: Distribution of PBMC population variables**

| Cell population | Distribution |
|---|---|
| CD3+ T cells %Parent | Non-normal |
| CD3+CD4+CD8+ T cells %Parent | Non-normal |
| CD3+CD4+ T cells %Parent | Normal |
| CD3+CD4+HLA-DR+ T cells MFI | Non-normal |
| CD3+CD4+CD45RA-CCR7+ T cells %Parent | Normal |
| CD3+CD4+CD45RA+CCR7+ T cells %Parent | Non-normal |
| CD3+CD4+CD45RA-CCR7- T cells %Parent | Non-normal |
| CD3+CD4+CD45RA+CCR7- T cells %Parent | Non-normal |
| CD3+CD8+ T cells %Parent | Non-normal |
| CD3+CD8+HLA-DR+ T cells MFI | Non-normal |
| CD3+CD8+CD45RA-CCR7+ T cells %Parent | Non-normal |
| CD3+CD8+CD45RA+CCR7+ T cells %Parent | Non-normal |
| CD3+CD8+CD45RA-CCR7- T cells %Parent | Non-normal |
| CD3+CD8+CD45RA+CCR7- T cells %Parent | Normal |
| CD19+ B cells %Parent | Non-normal |
| CD19+HLA-DR+ B cells MFI | Normal |
| CD14+ Monocytes %Parent | Non-normal |
| CD14+HLA-DR+ Monocytes MFI | Non-normal |

All cell populations were measured as a percentage of parent population
Activated cell populations were measured as mean fluorescent intensity (MFI)
CD = Cluster of differentiation
HLA-DR = Human leukocyte antigen – D receptor
CCR = Chemokine receptor

ANOVA testing on normally distributed variables revealed statistically significant differences between groups for CD4+ T cells (F=3.209, P=0.046) and CD19+HLA-DR+ B cells (F=4.786, P=0.011). Post-hoc analysis revealed that CD4+ T cells were significantly lower in the DLB group compared with AD (P=0.043), and that CD19+HLA-DR+ B cells were significantly lower in the DLB group compared with AD (P=0.009).

Linear regression analysis with CD4+ T cells as the dependent variable showed that the difference between DLB and AD groups remained significant (mean difference 11.6 (95% 1.8-21.4), t test P=0.022) after correction for age and gender as possible confounders. Linear regression with CD19+HLA-DR+ B cells showed that the difference between DLB and AD groups also remained significant (mean difference 13164 (95% 4592-21736), t test P=0.003) after correction for age and gender.

All other variables were non-significant for group differences using Kruskal-Wallis testing. This included comparisons of all PBMC populations between the control group and either dementia group. A summary of data, including mean (SD) for normally distributed data and median (IQR) for non-normally distributed data, is shown in table 3.17.

**Table 3.17: PBMC subsets measured by flow cytometry in SILAD**

| PBMC subset | Controls | DLB | AD | P |
|---|---|---|---|---|
| CD3+ T cells | 64.2 (19.1) | 62.6 (14.8) | 59.1 (10.5) | 0.526 |
| CD4+CD8+CD3+ T cells | 0.4 (0.6) | 0.4 (0.9) | 0.3 (0.6) | 0.635 |
| CD4+ T cells | 57.9±16.7 | 49.6±19.5 | 61.1±14.9 | **0.046**[b] |
| CD4+HLA-DR+ MFI T cells | 10246 (8201) | 10128 (6106) | 9843 (4788) | 0.884 |
| CD4+CD45RA-CCR7+ T cells | 18.5±8.1 | 20.6±9.3 | 19.5±10.8 | 0.710 |
| CD4+CD45RA+CCR7+ T cells | 62.2 (31.6) | 60.4 (31.1) | 65.4 (32.6) | 0.415 |
| CD4+CD45RA-CCR7- T cells | 12.6 (18.2) | 11.3 (16.8) | 12.4 (13.0) | 0.567 |
| CD4+CD45RA+CCR7- T cells | 1.8 (5.0) | 2.5 (4.4) | 1.8 (2.9) | 0.699 |
| CD8+ T cells | 29.9 (13.3) | 40.1 (28.8) | 25.0 (17.7) | 0.053 |
| CD8+HLA-DR+ MFI T cells | 5321 (2190) | 5530 (3269) | 5879 (3968) | 0.804 |
| CD8+CD45RA-CCR7+ T cells | 5.3 (5.2) | 4.6 (4.5) | 5.4 (5.6) | 0.899 |
| CD8+CD45RA+CCR7+ T cells | 29.4 (27.5) | 21.8 (20.7) | 28.1 (34.7) | 0.552 |
| CD8+CD45RA-CCR7- T cells | 22.4 (20.1) | 19.0 (21.9) | 16.7 (18.1) | 0.360 |
| CD8+CD45RA+CCR7- T cells | 42.8±20.9 | 50.8±20.0 | 45.2±20.8 | 0.339 |
| CD19+ B cells | 4.7 (2.4) | 4.7 (4.8) | 5.5 (4.5) | 0.212 |
| CD19+HLA-DR+ MFI B cells | 61861±14832 | 53442±16729 | 66530±15324 | **0.011**[b] |
| CD14+ Monocytes | 15.0 (12.2) | 15.2 (13.5) | 17.0 (11.6) | 0.499 |
| CD14+HLA-DR+ MFI Monocytes | 29124 (10374) | 28697 (10298) | 31310 (9783) | 0.657 |

Results are presented as mean±SD or median (IQR) percentage of parent population, or (for HLA-DR) mean fluorescent intensity
MFI= Mean fluorescent intensity
P is P value using either ANOVA or Kruskal-Wallis test
*a* = significant post-hoc difference DLB vs CTL, after correction for age and gender
*b* = significant post-hoc difference DLB vs AD, after correction for age and gender
*c* = significant post-hoc difference AD vs CTL, after correction for age and gender

### 3.2.9 Serum cytokines

All serum cytokine concentrations were assessed for normality visually using QQ plots, with all ten cytokines demonstrating non-normally distributed data. The Kruskal-Wallis test was therefore used to check for group differences in cytokine concentration, with post-hoc analysis using Dunn-Bonferroni to test for significant pairwise differences (using adjusted significance values for

multiple tests). One serum concentration for IL1β was excluded for being an extreme outlier.

IL1β, IL6, IL10 and TNFα showed statistically significant differences between groups. A summary of data, test statistics and significance values is show in table 3.18 and illustrated in figure 3.4.

**Table 3.18: Serum cytokine concentrations in SILAD**

| Cytokine | Controls | DLB | AD | P |
|---|---|---|---|---|
| IL1β | 0.00 [0.00-0.00] | 0.04 [0.00-0.07] | 0.00 [0.00-0.00] | **14.068 (P=0.001)** |
| IL2 | 0.11 [0.00-0.30] | 0.25 [0.15-0.33] | 0.24 [0.13-0.35] | 4.162 (P=0.125) |
| IL4 | 0.09 [0.00-0.20] | 0.17 [0.13-0.21] | 0.16 [0.03-0.22] | 3.929 (P=0.140) |
| IL6 | 0.92 [0.60-1.34] | 1.35 [1.03-1.83] | 1.31 [0.93-1.54] | **10.007 (P=0.007)** |
| IL8 | 7.45 [6.27-11.08] | 6.22 [3.89-9.46] | 7.49 [5.58-11.86] | 3.640 (P=0.162) |
| IL10 | 0.41 [0.18-0.65] | 0.63 [0.48-0.77] | 0.62 [0.28-0.76] | **6.307 (P=0.043)** |
| IL12 | 0.21 [0.12-0.43] | 0.28 [0.20-0.43] | 0.29 [0.00-0.41] | 1.270 (P=0.530) |
| IL13 | 1.46 [0.00-2.23] | 2.23 [1.43-2.80] | 2.17 [0.00-2.84] | 4.623 (P=0.099) |
| TNFα | 1.51 [1.31-1.95] | 1.86 [1.51-2.74] | 1.82 [1.46-2.40] | **6.603 (P=0.037)** |
| IFNγ | 3.70 [3.00-6.02] | 4.74 [2.76-10.70] | 6.50 [3.99-8.98] | 5.013 (P=0.082) |

Results are presented as median pg/ml (IQR)
P is Kruskal-Wallis test statistic (P value)

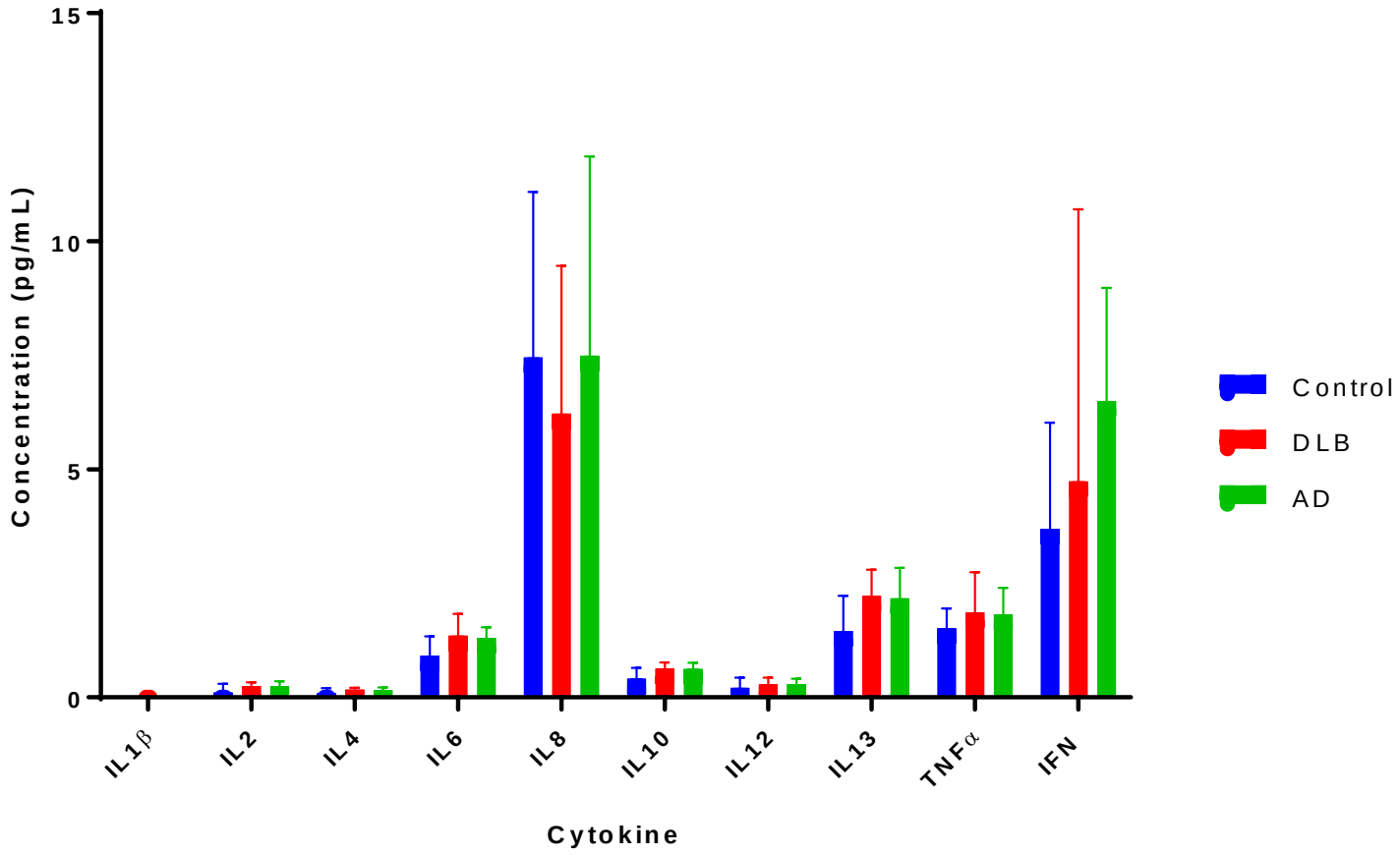


**Figure 3.4: Grouped bar chart of serum cytokine concentrations in SILAD**
Data presented as median serum concentrations and error bars represent interquartile range

Post-hoc analysis revealed the following results:

- IL1β concentration was significantly higher in DLB than in AD (P=0.005) and in controls (P=0.002).
- IL6 concentration was significantly higher in DLB than in controls (P=0.006).
- IL10 concentration was significantly higher in DLB than in controls (P=0.048).
- TNFα concentration was significantly higher in DLB than in controls (P=0.036).

However, the above tests did not adjust for age and gender as possible confounders, so further statistical analysis was performed. A large proportion of IL1β concentrations were below the lower limit of detection and recorded as 0. Therefore, this variable was re-coded as a binary value – 0 indicating that IL1β was not present and 1 indicating the presence of IL1β. This revealed that a detectable concentration of IL1β was present in 16.1% of the control group, 53.3% of the DLB group and 16.1% of the AD group. There was statistically significantly different IL1β concentrations between groups, the highest being found in the DLB group, as determined by Pearson Chi-squared test ($\chi^2(2)=13.803$, P=0.001). Logistic regression showed that the difference between the control and DLB groups remained unchanged (P=0.022), as did the difference between AD and DLB groups (P=0.004), after correction for age and gender. Although not statistically significant, it was noteworthy that the number of patients who had infections in the preceding 6 weeks was higher in the DLB group than in both AD and control groups. Further logistic regression analysis to correct for presence of recent infections, in addition to age and gender, showed that the difference in serum IL1β between DLB and AD groups remained significant (P=0.005), but the difference between DLB and control groups lost significance (P=0.077).

Data for serum IL6, IL10 and TNFα was log10 transformed to allow correction for potential confounders. Logged data for these three variables were found to be normally distributed using QQ plots. Linear regression analysis showed that serum IL6 remained significantly higher in the DLB group than controls (P=0.015) after correction for age and gender. Further linear regression analysis revealed that serum IL6 remained significantly higher in the DLB group

compared with controls, after additionally correcting for infections in the preceding 6 weeks (P=0.041).

For TNFα, linear regression analysis showed that the difference between control and DLB groups was no longer significant (P=0.059) after correction for age and gender. Similarly, for IL10 linear regression showed that the difference between control and DLB groups lost significance (P=0.177) once corrected for age and gender.

In summary, after adjusting for age and gender, the following statistically significant results were found for serum cytokine concentrations:

- IL1β concentration was significantly higher in DLB than in AD (P=0.004) and controls (P=0.022)
- IL6 concentration was significantly higher in DLB than in controls (P=0.015).

### 3.2.10 Stimulation studies

Cytokine concentrations in the supernatant of stimulated PBMC was divided by the concentration in unstimulated samples for each case. This provided a ratio indicating the level of increased cytokine production attributable to stimulation. Ratios for both LPS and PHA stimulated cytokine concentrations were assessed for normality using QQ plots. All ratios were deemed non-normal in their distribution and therefore the Kruskal-Wallis test was employed to test for group differences.

No stimulated cytokine ratios were found to be significantly different between the three groups. Further post-hoc analysis was therefore not performed. Table 3.19 shows a summary of data, with Kruskal-Wallis test statistics and significance values.

**Table 3.19: Stimulated cytokine ratios in SILAD**

| Cytokine | Controls | DLB | AD | P |
|---|---|---|---|---|
| IL1β - LPS | 898.34 (2086.08) | 465.96 (846.276) | 635.33 (1554.50) | 3.678, P=0.159 |
| IL1β - PHA | 819.50 (1634.54) | 418.57 (578.34) | 499.16 (1641.01) | 4.207, P=0.122 |
| IL2 - LPS | 3.94 (5.58) | 3.71 (2.72) | 4.60 (3.66) | 1.891, P=0.388 |
| IL2 - PHA | 4.43 (4.89) | 3.24 (1.48) | 4.57 (4.25) | 4.220, P=0.121 |
| IL4 - LPS | 20.91 (21.54) | 13.00 (14.64) | 22.22 (24.61) | 5.128, P=0.077 |
| IL4 - PHA | 23.03 (28.51) | 13.77 (8.51) | 17.31 (24.17) | 3.287, P=0.193 |
| IL6 - LPS | 245.95 (450.90) | 140.50 (295.15) | 168.36 (385.71) | 4.130, P=0.127 |
| IL6 - PHA | 231.47 (504.84) | 123.75 (315.41) | 144.93 (296.03) | 3.699, P=0.157 |
| IL8 - LPS | 8.42 (8.82) | 5.52 (4.95) | 7.56 (9.68) | 3.122, P=0.210 |
| IL8 - PHA | 7.50 (8.50) | 4.93 (3.69) | 6.36 (10.64) | 1.919, P=0.383 |
| IL10 – LPS | 39.32 (85.95) | 113.68 (80.70) | 62.74 (68.15) | 2.344, P=0.310 |
| IL10 – PHA | 43.50 (78.72) | 25.55 (57.80) | 34.11 (91.33) | 2.042, P=0.360 |
| IL12 – LPS | 14.33 (19.45) | 14.05 (16.97) | 18.23 (17.76) | 2.265, P=0.322 |
| IL12 – PHA | 15.02 (20.93) | 11.67 (17.07) | 13.97 (17.28) | 1.269, P=0.530 |
| IL13 - LPS | 5.85 (3.60) | 4.46 (1.65) | 5.74 (3.82) | 5.152, P=0.076 |
| IL13 - PHA | 5.43 (5.25) | 4.55 (1.49) | 6.12 (4.45) | 4.805, P=0.091 |
| TNFα - LPS | 94.43 (73.46) | 62.64 (74.23) | 69.00 (126.24) | 1.872, P=0.392 |
| TNFα - PHA | 115.24 (135.71) | 74.49 (67.25) | 78.30 (172.48) | 2.405, P=0.300 |
| IFNγ - LPS | 99.32 (301.99) | 82.24 (137.06) | 156.35 (170.42) | 2.943, P=0.230 |
| IFNγ - PHA | 197.17 (415.08) | 106.00 (217.47) | 167.35 (277.66) | 1.676, P=0.433 |

Results are presented as ratio of stimulated cytokine concentration divided by unstimulated cytokine concentration
P is Kruskal-Wallis test statistic, P value

### 3.2.11 Inflammation and clinical features in DLB

Since all neuropsychiatric tests were deemed to be non-normal in their distribution, Spearman's rank correlation testing was used to assess for significant associations between markers of inflammation and the clinical features of DLB. $P<0.01$ was selected for significance to account for multiple comparisons. AD and control cases were excluded from this analysis.

Firstly, PBMC populations were tested for significant associations with selected neuropsychiatric tests (MoCA, CAF, UPDRS, NPI delusions and NPI hallucinations). These tests were selected for comparison due to their clinical relevance to DLB. Table 3.20 (divided over two pages) shows correlation coefficients and significance values for these comparisons. No significant associations were found between any selected neuropsychiatric test score and any PBMC population in DLB.

Secondly, serum cytokine concentrations were tested for significant association with the neuropsychiatric test scores above. Table 3.21 shows correlation coefficients and significance values for these comparisons. No significant associations were found between any selected neuropsychiatric test score and any serum concentration in DLB.

Lastly, stimulated cytokine ratios were tested for significant association with the neuropsychiatric test scores above. Tables 3.22 and 3.23 show correlation co-efficients and significance values for these comparisons. No significant associations were found between any selected neuropsychiatric test score and any stimulated cytokine ratio in DLB.

In summary, no significant associations were found for any markers of peripheral inflammation and any of the clinical features of DLB.

**Table 3.20: Correlations between PBMC subsets and clinical features in DLB**

| PBMC population | MoCA | FCSRT-IR total | CSDD | CAF | UPDRS total | UPDRS motor | NPI total | NPI delusions | NPI hallucination |
|---|---|---|---|---|---|---|---|---|---|
| CD3+ T cells | -0.255 | -0.128 | 0.153 | -0.017 | 0.203 | 0.140 | 0.145 | -0.029 | 0.268 |
| Sig. (two-tailed) | 0.199 | 0.533 | 0.447 | 0.931 | 0.309 | 0.487 | 0.472 | 0.884 | 0.176 |
| CD3+CD4+CD8+ T cells | 0.243 | 0.084 | 0.122 | -.460* | -0.235 | -0.251 | -0.133 | -0.067 | -0.101 |
| Sig. (two-tailed) | 0.223 | 0.683 | 0.544 | 0.016 | 0.237 | 0.207 | 0.508 | 0.740 | 0.618 |
| CD4+ T cells | 0.112 | -0.084 | 0.267 | -0.331 | -0.109 | -0.072 | 0.019 | -0.140 | 0.002 |
| Sig. (two-tailed) | 0.580 | 0.683 | 0.178 | 0.092 | 0.589 | 0.720 | 0.924 | 0.487 | 0.990 |
| CD4+HLA-DR+ T cells | -0.194 | -0.253 | 0.145 | -0.001 | -0.292 | -0.337 | 0.290 | .421* | 0.182 |
| Sig. (two-tailed) | 0.333 | 0.212 | 0.471 | 0.998 | 0.139 | 0.086 | 0.143 | 0.029 | 0.364 |
| CD4+CD45RA-CCR7+ T cells | -0.048 | 0.023 | -0.079 | -0.164 | -0.075 | -0.042 | 0.020 | -0.063 | 0.005 |
| Sig. (two-tailed) | 0.812 | 0.912 | 0.697 | 0.413 | 0.709 | 0.837 | 0.923 | 0.753 | 0.980 |
| CD4+CD45RA+CCR7+ T cells | 0.072 | -0.144 | 0.222 | 0.129 | 0.108 | 0.046 | -0.034 | -0.123 | -0.194 |
| Sig. (two-tailed) | 0.722 | 0.482 | 0.266 | 0.521 | 0.592 | 0.819 | 0.865 | 0.541 | 0.331 |
| CD4+CD45RA-CCR7- T cells | -0.026 | 0.102 | -0.174 | -0.236 | -0.140 | -0.089 | 0.067 | 0.222 | 0.273 |
| Sig. (two-tailed) | 0.899 | 0.619 | 0.385 | 0.237 | 0.487 | 0.658 | 0.739 | 0.266 | 0.168 |
| CD4+CD45RA+CCR7- T cells | -0.014 | 0.033 | -0.183 | -0.014 | -0.033 | -0.058 | -0.005 | 0.176 | 0.055 |
| Sig. (two-tailed) | 0.944 | 0.873 | 0.361 | 0.946 | 0.871 | 0.772 | 0.980 | 0.380 | 0.784 |
| CD8+ T cells | -0.059 | 0.127 | -0.202 | 0.237 | 0.086 | 0.064 | -0.123 | 0.026 | -0.045 |
| Sig. (two-tailed) | 0.768 | 0.537 | 0.313 | 0.234 | 0.671 | 0.753 | 0.542 | 0.896 | 0.825 |

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| PBMC population | MoCA | FCSRT-IR total | CSDD | CAF | UPDRS total | UPDRS motor | NPI total | NPI delusions | NPI hallucination |
|---|---|---|---|---|---|---|---|---|---|
| CD8+HLA-DR+ T cells | -0.112 | -0.332 | 0.065 | -0.129 | -0.062 | -0.145 | -0.003 | 0.128 | 0.164 |
| Sig. (two-tailed) | 0.580 | 0.097 | 0.746 | 0.520 | 0.757 | 0.470 | 0.988 | 0.523 | 0.413 |
| CD8+CD45RA-CCR7+ T cells | 0.182 | 0.085 | 0.178 | -0.259 | -0.217 | -0.155 | -0.164 | -0.212 | -0.148 |
| Sig. (two-tailed) | 0.364 | 0.681 | 0.373 | 0.192 | 0.278 | 0.439 | 0.412 | 0.288 | 0.462 |
| CD8+CD45RA+CCR7+ T cells | -0.027 | -0.148 | 0.159 | -0.050 | 0.066 | 0.041 | -0.008 | -0.132 | -0.129 |
| Sig. (two-tailed) | 0.894 | 0.472 | 0.429 | 0.805 | 0.745 | 0.838 | 0.967 | 0.512 | 0.520 |
| CD8+CD45RA-CCR7- T cells | 0.246 | 0.327 | 0.247 | -0.229 | -0.190 | -0.087 | -0.001 | -0.117 | -0.267 |
| Sig. (two-tailed) | 0.216 | 0.103 | 0.214 | 0.251 | 0.341 | 0.666 | 0.998 | 0.562 | 0.179 |
| CD8+CD45RA+CCR7- T cells | -0.096 | -0.070 | -0.335 | 0.136 | 0.046 | 0.011 | 0.028 | 0.144 | 0.313 |
| Sig. (two-tailed) | 0.635 | 0.735 | 0.088 | 0.498 | 0.819 | 0.957 | 0.891 | 0.472 | 0.111 |
| CD19+ B cells | 0.108 | -0.048 | 0.121 | -0.104 | -0.263 | -0.251 | 0.240 | 0.195 | 0.117 |
| Sig. (two-tailed) | 0.591 | 0.817 | 0.547 | 0.607 | 0.186 | 0.207 | 0.229 | 0.329 | 0.561 |
| CD19+HLA-DR+ B cells | 0.045 | -0.006 | 0.052 | -0.233 | -0.250 | -0.291 | 0.070 | -0.026 | -0.163 |
| Sig. (two-tailed) | 0.825 | 0.978 | 0.797 | 0.243 | 0.209 | 0.140 | 0.729 | 0.896 | 0.418 |
| CD14+ Monocytes | 0.008 | -0.107 | 0.057 | -0.269 | -0.038 | -0.113 | -0.137 | -0.047 | -0.164 |
| Sig. (two-tailed) | 0.967 | 0.602 | 0.779 | 0.175 | 0.851 | 0.576 | 0.495 | 0.816 | 0.414 |
| CD14+HLA-DR+ Monocytes | -0.001 | 0.088 | 0.247 | -0.201 | -0.191 | -0.252 | -0.163 | -0.230 | -0.287 |
| Sig. (two-tailed) | 0.996 | 0.668 | 0.215 | 0.314 | 0.341 | 0.204 | 0.416 | 0.249 | 0.146 |

Results are Spearman's rank correlations
*P<0.05, **P<0.01 and in bold

**Table 3.21: Correlations between serum cytokine concentrations and clinical features in DLB**

| Serum concentration | | MoCA | FCSRT-IR total | CSDD | CAF | UPDRS total | UPDRS motor | NPI total | NPI delusions | NPI hallucinations |
|---|---|---|---|---|---|---|---|---|---|---|
| IL1β | | .392* | .421* | -0.123 | -0.090 | -0.022 | 0.014 | -0.102 | -0.248 | -0.124 |
| | Sig. (two-tailed) | 0.032 | 0.023 | 0.517 | 0.636 | 0.908 | 0.943 | 0.591 | 0.187 | 0.515 |
| IL2 | | -0.323 | -0.182 | .373* | 0.205 | 0.327 | 0.240 | 0.226 | -0.065 | 0.247 |
| | Sig. (two-tailed) | 0.076 | 0.336 | 0.039 | 0.270 | 0.072 | 0.193 | 0.221 | 0.727 | 0.180 |
| IL4 | | -0.336 | -0.155 | 0.241 | 0.264 | 0.147 | 0.031 | 0.125 | -0.059 | 0.236 |
| | Sig. (two-tailed) | 0.064 | 0.413 | 0.191 | 0.151 | 0.430 | 0.870 | 0.502 | 0.753 | 0.201 |
| IL6 | | -0.311 | -0.260 | 0.212 | 0.083 | 0.235 | 0.138 | -0.022 | -0.051 | 0.061 |
| | Sig. (two-tailed) | 0.089 | 0.166 | 0.253 | 0.655 | 0.204 | 0.458 | 0.905 | 0.786 | 0.743 |
| IL8 | | -0.022 | -0.271 | 0.172 | -0.172 | 0.003 | -0.077 | 0.137 | 0.052 | 0.347 |
| | Sig. (two-tailed) | 0.906 | 0.147 | 0.355 | 0.353 | 0.986 | 0.679 | 0.463 | 0.780 | 0.056 |
| IL10 | | -0.024 | -0.009 | 0.073 | 0.026 | 0.134 | 0.040 | -0.013 | 0.072 | 0.134 |
| | Sig. (two-tailed) | 0.900 | 0.964 | 0.698 | 0.889 | 0.472 | 0.832 | 0.943 | 0.699 | 0.473 |
| IL12 | | -.377* | -0.218 | 0.270 | 0.249 | 0.071 | -0.052 | 0.124 | -0.034 | 0.206 |
| | Sig. (two-tailed) | 0.037 | 0.247 | 0.141 | 0.177 | 0.703 | 0.783 | 0.505 | 0.856 | 0.266 |
| IL13 | | -0.280 | -0.098 | 0.193 | 0.177 | 0.143 | 0.014 | 0.128 | 0.055 | 0.183 |
| | Sig. (two-tailed) | 0.127 | 0.606 | 0.298 | 0.342 | 0.441 | 0.941 | 0.493 | 0.768 | 0.325 |
| TNFα | | -0.236 | -0.254 | -0.007 | -0.120 | 0.258 | 0.198 | -0.127 | 0.068 | 0.319 |
| | Sig. (two-tailed) | 0.201 | 0.176 | 0.971 | 0.522 | 0.161 | 0.287 | 0.497 | 0.717 | 0.080 |
| IFNγ | | -.358* | -0.089 | -0.331 | 0.265 | 0.283 | 0.173 | -0.272 | -0.078 | 0.009 |
| | Sig. (two-tailed) | 0.048 | 0.640 | 0.069 | 0.150 | 0.122 | 0.351 | 0.139 | 0.675 | 0.960 |

Results are Spearman's rank correlations
*P<0.05, **P<0.01 and in bold

**Table 3.22: Correlations between LPS stimulated cytokine ratios and clinical features in DLB**

| LPS stimulated ratio | | MoCA | FCSRT-IR total | CSDD | CAF | UPDRS total | UPDRS motor | NPI total | NPI delusions | NPI hallucinations |
|---|---|---|---|---|---|---|---|---|---|---|
| IL1β | | 0.051 | -0.161 | -0.167 | -0.070 | 0.202 | 0.219 | -0.102 | 0.028 | -0.100 |
| | Sig. (two-tailed) | 0.788 | 0.405 | 0.377 | 0.713 | 0.285 | 0.244 | 0.593 | 0.883 | 0.600 |
| IL2 | | 0.124 | 0.082 | -0.123 | -0.037 | 0.035 | 0.145 | -0.146 | 0.132 | -0.335 |
| | Sig. (two-tailed) | 0.514 | 0.672 | 0.516 | 0.845 | 0.855 | 0.445 | 0.440 | 0.488 | 0.070 |
| IL4 | | 0.045 | -0.065 | -0.275 | -0.051 | 0.111 | 0.210 | -0.229 | 0.092 | -0.242 |
| | Sig. (two-tailed) | 0.814 | 0.738 | 0.141 | 0.790 | 0.558 | 0.266 | 0.224 | 0.630 | 0.198 |
| IL6 | | 0.237 | -0.197 | 0.008 | -0.230 | 0.053 | 0.060 | 0.018 | 0.205 | 0.020 |
| | Sig. (two-tailed) | 0.208 | 0.306 | 0.965 | 0.221 | 0.781 | 0.751 | 0.924 | 0.278 | 0.916 |
| IL8 | | 0.222 | -0.026 | -0.092 | -0.163 | 0.029 | 0.126 | -0.232 | -0.022 | -.374* |
| | Sig. (two-tailed) | 0.238 | 0.891 | 0.628 | 0.389 | 0.877 | 0.506 | 0.217 | 0.909 | 0.042 |
| IL10 | | 0.060 | -0.043 | -0.102 | -0.204 | 0.204 | 0.221 | -0.073 | 0.170 | -0.145 |
| | Sig. (two-tailed) | 0.753 | 0.825 | 0.593 | 0.279 | 0.280 | 0.241 | 0.700 | 0.370 | 0.443 |
| IL12 | | -0.014 | -0.014 | -0.291 | 0.123 | 0.280 | 0.351 | -0.316 | -0.079 | -0.188 |
| | Sig. (two-tailed) | 0.942 | 0.943 | 0.119 | 0.519 | 0.134 | 0.057 | 0.089 | 0.677 | 0.319 |
| IL13 | | 0.116 | 0.116 | -0.081 | 0.092 | 0.116 | 0.244 | -0.069 | 0.039 | -.420* |
| | Sig. (two-tailed) | 0.541 | 0.550 | 0.671 | 0.629 | 0.540 | 0.193 | 0.718 | 0.838 | 0.021 |
| TNFα | | 0.007 | -0.074 | -0.153 | 0.011 | 0.288 | 0.319 | -0.190 | 0.007 | -0.186 |
| | Sig. (two-tailed) | 0.970 | 0.702 | 0.421 | 0.953 | 0.123 | 0.086 | 0.315 | 0.971 | 0.324 |
| IFNγ | | 0.090 | 0.029 | -0.296 | -0.100 | 0.118 | 0.133 | -0.278 | 0.074 | -0.310 |
| | Sig. (two-tailed) | 0.636 | 0.882 | 0.112 | 0.599 | 0.534 | 0.484 | 0.136 | 0.697 | 0.095 |

Results are Spearman's rank correlations
*P<0.05, **P<0.01 and in bold

**Table 3.23: Correlations between PHA stimulated cytokine ratios and clinical features in DLB**

| PHA stimulated ratio | | MoCA | FCSRT-IR total | CSDD | CAF | UPDRS total | UPDRS motor | NPI total | NPI delusions | NPI hallucinations |
|---|---|---|---|---|---|---|---|---|---|---|
| IL1β | | -0.143 | -0.305 | -0.142 | -0.114 | 0.257 | 0.185 | -0.023 | 0.106 | -0.041 |
| | Sig. (two-tailed) | 0.452 | 0.108 | 0.454 | 0.549 | 0.170 | 0.329 | 0.905 | 0.577 | 0.829 |
| IL2 | | 0.040 | -0.010 | -0.140 | -0.188 | 0.027 | 0.052 | -0.207 | 0.006 | -.368* |
| | Sig. (two-tailed) | 0.836 | 0.960 | 0.461 | 0.321 | 0.889 | 0.784 | 0.272 | 0.976 | 0.045 |
| IL4 | | 0.022 | -0.078 | -0.282 | -0.077 | 0.061 | 0.143 | -0.278 | 0.064 | -0.320 |
| | Sig. (two-tailed) | 0.908 | 0.688 | 0.131 | 0.686 | 0.748 | 0.450 | 0.137 | 0.738 | 0.085 |
| IL6 | | 0.222 | -0.190 | 0.014 | -0.260 | 0.017 | 0.023 | 0.034 | 0.220 | 0.027 |
| | Sig. (two-tailed) | 0.238 | 0.324 | 0.940 | 0.165 | 0.930 | 0.902 | 0.860 | 0.242 | 0.889 |
| IL8 | | 0.202 | -0.029 | -0.095 | -0.160 | -0.063 | 0.028 | -0.218 | 0.008 | -0.346 |
| | Sig. (two-tailed) | 0.285 | 0.880 | 0.617 | 0.398 | 0.739 | 0.885 | 0.247 | 0.967 | 0.061 |
| IL10 | | -0.101 | -0.129 | 0.021 | -0.245 | 0.146 | 0.111 | -0.066 | 0.072 | -0.160 |
| | Sig. (two-tailed) | 0.594 | 0.506 | 0.911 | 0.192 | 0.441 | 0.560 | 0.729 | 0.706 | 0.399 |
| IL12 | | -0.044 | -0.005 | -0.263 | 0.088 | 0.273 | 0.325 | -0.331 | -0.144 | -0.266 |
| | Sig. (two-tailed) | 0.817 | 0.980 | 0.161 | 0.645 | 0.145 | 0.080 | 0.074 | 0.448 | 0.155 |
| IL13 | | -0.037 | 0.098 | -0.077 | 0.172 | 0.093 | 0.180 | -0.171 | -0.115 | -.416* |
| | Sig. (two-tailed) | 0.847 | 0.611 | 0.687 | 0.363 | 0.625 | 0.341 | 0.366 | 0.544 | 0.022 |
| TNFα | | -0.083 | -0.127 | -0.221 | 0.008 | 0.342 | 0.352 | -0.176 | -0.086 | -0.073 |
| | Sig. (two-tailed) | 0.664 | 0.513 | 0.241 | 0.968 | 0.064 | 0.056 | 0.354 | 0.650 | 0.703 |
| IFNγ | | 0.028 | 0.012 | -0.120 | -0.140 | 0.173 | 0.130 | -0.125 | -0.029 | -0.296 |
| | Sig. (two-tailed) | 0.882 | 0.950 | 0.529 | 0.462 | 0.360 | 0.493 | 0.509 | 0.879 | 0.112 |

Results are Spearman's rank correlations
*P<0.05, **P<0.01 and in bold

### 3.2.12 Effect of *APOE* genotype

In order to examine the effect of *APOE* genotype on peripheral inflammatory markers, each group was assessed separately (controls, DLB and AD) using *APOE* genotype as the grouping variable. The non-parametric Mann-Whitney U test was used to test for group differences (ε4 carriers versus non-carriers). Differences were deemed significant when the P value was <0.05. The paragraphs below present findings from serum cytokine and stimulated cytokine ratios in controls, DLB and AD. Stimulated cytokine ratios, split by *APOE* genotype, are summarised in tables 3.24 (LPS stimulated) and 3.25 (PHA stimulated).

In controls, no significant differences were found in any serum cytokine concentration or any stimulated PBMC cytokine ratio between ε4 carriers and non-carriers.

In DLB, the only significant difference between groups was for serum IL2 concentration, which was higher in ε4 carriers (mean rank 19.47) compared with non-carriers (mean rank 12.75) as demonstrated by Mann-Whitney U test (MWU=172, P=0.041). There were no significant differences between ε4 carriers and non-carriers for any other serum cytokine concentration or any stimulated PBMC cytokine ratio.

In AD, there were no significant differences between ε4 carriers and non-carriers in any serum cytokine concentrations. However, numerous stimulated cytokine ratios were significantly lower in ε4 carriers compared with non-carriers, as listed below.

- LPS stimulated ε4 carriers vs non carriers
    - IL1β (mean rank 12.11 vs 19.56, MWU=40, P=0.025)
    - IL4 (mean rank 12.32 vs 19.11, MWU=44, P=0.041)
    - IL6 (mean rank 12.32 vs 19.11, MWU=44, P=0.041)
    - TNFα (mean rank 12.16 vs 19.44, MWU=41, P=0.029)
- PHA stimulated ε4 carriers vs non carriers
    - IL1β (mean rank 12.05 vs 19.67, MWU=39, P=0.022)
    - IL4 (mean rank 11.63 vs 20.56, MWU=31, P=0.007)

- IL6 (mean rank 12.11 vs 19.56, MWU=40, P=0.025)
- IL12 (mean rank 12.05 vs 19.67, MWU=39, P=0.022)
- TNFα (mean rank 12.11 vs 19.56, MWU=40, P=0.025)

The above significant results in the AD group were subject to further statistical analysis to correct for age and gender as potential confounders. All stimulated cytokine ratios were log10 transformed and then assessed for normality using QQ plots. All transformed variables were deemed to be normally distributed. Linear regression was then used to adjust for age and gender, with significance values shown below.

- LPS stimulated ε4 carriers vs non carriers
  - IL1β P=0.048
  - IL4 P=0.076
  - IL6 P=0.062
  - TNFα P=0.048
- PHA stimulated ε4 carriers vs non carriers
  - IL1β P=0.034
  - IL4 P=0.016
  - IL6 P=0.032
  - IL12 P=0.046
  - TNFα P=0.022

Therefore, in AD, stimulated PBMC cytokine ratios were significantly lower in ε4 carriers for LPS stimulated IL1β and TNFα, and for PHA stimulated IL1β, IL4, IL6, IL12 and TNFα.

**Table 3.24: LPS stimulated cytokine ratios by *APOE* genotype**

| LPS stimulated | Controls | DLB | AD |
|---|---|---|---|
| IL1β – *APOE* ε4- | 904.91 (1488.24) | 694.07 (1038.16) | **1983.00 (4464.76)** * |
| IL1β – *APOE* ε4+ | 855.35 (4428.61) | 313.29 (381.66) | **487.59 (895.27)** * |
| IL2 – *APOE* ε4- | 4.08 (4.68) | 3.71 (3.13) | 5.11 (3.40) |
| IL2 - *APOE* ε4+ | 3.29 (9.42) | 3.74 (1.88) | 4.57 (4.52) |
| IL4 - *APOE* ε4- | 20.24 (21.70) | 14.80 (21.51) | **32.20 (77.72)** * |
| IL4 - *APOE* ε4+ | 29.03 (79.41) | 11.86 (13.13) | **20.53 (20.59)** * |
| IL6 - *APOE* ε4- | 238.62 (486.06) | 141.90 (250.26) | **267.53 (865.85)** * |
| IL6 - *APOE* ε4+ | 311.12 (962.81) | 108.73 (372.89) | **129.36 (133.41)** * |
| IL8 - *APOE* ε4- | 7.92 (9.21) | 6.36 (10.50) | 8.26 (19.79) |
| IL8 - *APOE* ε4+ | 8.61 (30.25) | 4.64 (3.68) | 6.32 (8.07) |
| IL10 – *APOE* ε4- | 31.58 (81.66) | 35.77 (117.47) | 81.48 (111.56) |
| IL10 - *APOE* ε4+ | 77.30 (349.25) | 34.51 (75.87) | 50.26 (88.23) |
| IL12 – *APOE* ε4- | 11.34 (18.14) | 11.98 (26.55) | 26.91 (22.66) |
| IL12 – *APOE* ε4+ | 17.65 (44.59) | 14.38 (12.01) | 15.70 (19.35) |
| IL13 - *APOE* ε4- | 5.38 (3.15) | 4.37 (2.56) | 6.22 (4.08) |
| IL13 - *APOE* ε4+ | 8.59 (6.33) | 4.63 (1.28) | 5.67 (4.38) |
| TNFα - *APOE* ε4- | 90.60 (79.38) | 63.90 (96.05) | **172.58 (180.71)** * |
| TNFα - *APOE* ε4+ | 105.75 (133.77) | 58.88 (55.23) | **55.98 (93.07)** * |
| IFNγ - *APOE* ε4- | 99.02 (304.94) | 118.16 (336.32) | 233.32 (457.22) |
| IFNγ - *APOE* ε4+ | 148.87 (559.60) | 46.21 (88.22) | 126.82 (113.82) |

Results are presented as ratios of stimulated cytokine concentration – median (IQR)
P is Mann-Whitney U test statistic, P value
*P<0.05
**P<0.01

**Table 3.25: PHA stimulated cytokine ratios by *APOE* genotype**

| PHA stimulated | Controls | DLB | AD |
|---|---|---|---|
| IL1β – *APOE* ε4- | 878.63 (1351.86) | 538.64 (1045.61) | **1297.57 (3105.92)** * |
| IL1β – *APOE* ε4+ | 819.50 (3448.21) | 408.13 (394.85) | **289.82 (742.13)** * |
| IL2 – *APOE* ε4- | 3.74 (4.70) | 3.21 (3.50) | 4.64 (3.57) |
| IL2 - *APOE* ε4+ | 4.99 (25.80) | 3.50 (1.45) | 4.39 (4.39) |
| IL4 - *APOE* ε4- | 19.74 (29.51) | 17.23 (11.58) | **35.07 (85.64)** ** |
| IL4 - *APOE* ε4+ | 25.89 (140.20) | 12.11 (12.28) | **13.78 (17.89)** ** |
| IL6 - *APOE* ε4- | 201.11 (470.71) | 123.75 (242.52) | **269.57 (775.13)** * |
| IL6 - *APOE* ε4+ | 287.68 (773.84) | 110.70 (351.93) | **86.08 (116.63)** * |
| IL8 - *APOE* ε4- | 7.01 (8.55) | 5.95 (8.27) | 7.98 (23.27) |
| IL8 - *APOE* ε4+ | 8.76 (40.82) | 4.36 (2.48) | 4.55 (4.89) |
| IL10 – *APOE* ε4- | 34.24 (65.22) | 29.48 (76.37) | 89.43 (115.12) |
| IL10 – *APOE* ε4+ | 61.47 (456.39) | 20.76 (29.70) | 30.96 (84.31) |
| IL12 – *APOE* ε4- | 14.62 (21.86) | 11.42 (20.91) | **23.00 (27.71)** * |
| IL12 – *APOE* ε4+ | 16.46 (68.36) | 12.56 (11.90) | **12.22 (11.30)** * |
| IL13 - *APOE* ε4- | 5.08 (5.68) | 4.62 (1.70) | 8.15 (5.56) |
| IL13 - *APOE* ε4+ | 8.32 (9.48) | 4.55 (1.38) | 5.75 (4.01) |
| TNFα - *APOE* ε4- | 102.27 (127.61) | 72.44 (80.64) | **177.10 (157.56)** * |
| TNFα - *APOE* ε4+ | 141.68 (116.04) | 74.49 (58.17) | **59.96 (135.26)** * |
| IFNγ - *APOE* ε4- | 184.96 (414.27) | 129.05 (185.49) | 219.79 (546.61) |
| IFNγ - *APOE* ε4+ | 209.37 (470.41) | 96.53 (239.12) | 137.28 (281.50) |

Results are presented as ratios of stimulated cytokine concentration – median (IQR)
P is Mann-Whitney U test statistic, P value
*P<0.05
**P<0.01

## 3.3 Conclusions

The aim of this study was to define the phenotype of peripheral immune cells and serum markers of inflammation in DLB, AD and controls. An additional aim was to determine whether peripheral markers of inflammation were associated with the clinical features of DLB. SILAD was a cross-sectional, observational trial that involved participants undergoing a single study visit for neuropsychiatric testing and phlebotomy.

### 3.3.1 Clinical data

Assessment of baseline characteristics of SILAD participants revealed that the control group was significantly younger than both dementia groups, and therefore any significant results found between groups were corrected for age. Although not significant, the DLB group included more males than the AD group and controls. Due to the importance of gender as a biological variable, this was also corrected for during statistical analysis. Indeed, both of these variables have been shown to affect blood concentrations of cytokines and levels of PBMC stimulated cytokines [374-376]. The DLB, AD and control groups were otherwise well matched for years of education and disease duration. Similar numbers of participants were taking anti-inflammatory medications across the three groups, with the majority on low-dose aspirin for prophylaxis of cardiovascular disease.

The DLB group scored significantly higher in scales measuring the severity of the core features of DLB, namely hallucinations, fluctuations and Parkinsonism. This was expected as these patients would have been diagnosed with DLB based on the prominence of those very symptoms [18]. It is also noteworthy that the DLB group experienced a higher number of traumatic events in the six weeks preceding the study visit, possibly related to the number of falls, which is also a suggestive clinical feature of DLB. Use of antipsychotics and antiparkinsonian medications was also more prevalent in the DLB group, presumably as treatments for visual hallucinations and Parkinsonism. Interestingly, the DLB group also possessed higher carer distress scores than the AD group. Although not statistically significant, the finding of higher levels of carer distress in DLB does fit with previous literature [155, 156].

*APOE* genotype has been confirmed as a strong risk factor for DLB [38], although not as strong as it is for AD [105]. This study showed rates of possession of the *APOE* ε4 allele to be consistent with previous literature, with ε4 carriers accounting for less than one third of the control group, approximately one half of the DLB group and around two thirds of the AD group [377, 378].

### 3.3.2 Flow cytometry data

To my knowledge, there have been no previous studies investigating PBMC subsets in DLB and therefore the findings presented from this work are novel.

Data from flow cytometry experiments revealed significantly lower proportions of CD4+ T cells in DLB compared with AD, with the mean value in the control group bisecting the two dementia groups. T cells that express CD4 are referred to as helper T cells and play a key role in modulating the immune response through secretion of cytokines. Specifically, helper T cells can recognise peptides presented by HLA-DR molecules on antigen-presenting cells, and activate both B cells and CD8+ T cells to stimulate humoral and cell-mediated immunity respectively. Further examination of helper T cell subsets, using CD45RA and CCR7, can help to gain an understanding of the different phenotypes of these cells, especially with regards to their stage of maturity or differentiation. Specifically, cell populations can be identified that demonstrate a naïve, effector memory, central memory or TEMRA phenotype. In SILAD, this analysis revealed no significant changes in helper T cell subsets. Therefore, the reduction in the helper T cell population in DLB appeared not to be driven by a shift in subsets, but by an overall decline in cell population. This finding is supported by previous literature in PD which also shows a reduction in helper T cells [362, 364]. However, results from SILAD do not support one of these studies, which found preferential loss of the naïve helper T cell subset in PD [362]. Unfortunately there is no previous literature in this area specific to DLB to allow direct comparisons with results from SILAD.

Several major subsets of effector helper T cells have been identified on the basis of their distinct cytokine secretion profile and immunomodulatory effects. These include Th1 cells that activate macrophages to destroy

intracellular bacteria, as well as regulatory T cells that supress T cell activity and help to prevent autoimmunity during immune responses [158]. It is not possible to determine the secretion profile, and thus subset, of effector helper T cells without examining intracellular cytokine concentrations. This was not performed in SILAD and therefore it cannot be categorically proven whether a specific phenotype of effector helper T cells was preferentially reduced in DLB.

In addition to helper T cell changes, SILAD also revealed significantly decreased activation of HLA-DR+ B cells in DLB compared with AD. B cells can be activated dependent upon, or independent of, helper T cell involvement. Once antigen has bound with a B cell, this is taken up by endocytosis and degraded into antigen fragments, which are expressed on the cell surface bound with HLA-DR [158]. Therefore, HLA-DR+ B cells represent an activated B cell phenotype. The reduction in B cell activation could demonstrate a reduced level of humoral immunity in DLB, perhaps secondary to an impaired proliferative response to infection. Again, this finding is supported by previous literature in PD that shows a reduction in B cells [364].

T cells expressing CD8, also known as cytotoxic T cells, play a crucial role in the defence against intracellular pathogens and in tumour surveillance. Activation of cytotoxic T cells functions to neutralise infected or malignant cells. Results from SILAD showed a trend towards increased cytotoxic T cells in DLB compared with controls, with the proportion of these cells lower in AD than in controls, suggesting a divergent profile. However, these differences were not found to be statistically significant. In support of this finding, several previous studies examining cytotoxic T cells in AD [246, 247] and PD [363] have shown no significant alteration in this cell subset. It is noteworthy that several older studies have demonstrated reduced cytotoxic T cell populations in AD [244, 245, 248], a finding which would fit with the trend found in SILAD. The increase in proportion of cytotoxic T cells in DLB, albeit not a statistically significant increase, implies that there may be a greater role for adaptive immune clearance of virally infected host cells in DLB. One may speculate whether people with DLB may have a higher prevalence of chronic viral infections such as cytomegalovirus, which is highly prevalent in older people and is known to cause a robust and sustained clonal expansion in cytotoxic T cells [379].

Examination of cytotoxic T cell subsets in SILAD also reveals some interesting results. The proportion of naïve cytotoxic T cells (CD8+CD45RA+CCR7+) was reduced in DLB, while the proportion of TEMRA cytotoxic T cells (CD8+CD45RA+CCR7-) was increased. The proportions of these two cell subsets were broadly unchanged in AD compared with controls. Accumulation of the TEMRA cytotoxic T cell population has been proposed as the hallmark of immunosenescence in aging, where adaptive immune cells have limited proliferative capacity but tend to secrete a wide range of cytokines following activation [372].

When comparing AD with DLB, it is certainly noteworthy that the mean values of helper T cells and activated B cells in AD were higher than controls, while the mean values in DLB were lower than controls. An increase of these two cell populations have been demonstrated previously in AD [243, 245]. Results from SILAD, and from previous literature, appear to support opposing phenotypes of the adaptive immune system in LBD and AD. This suggests that whilst these two diseases groups are both characterised by cerebral protein deposition, their peripheral adaptive immune phenotypes are markedly dissimilar. It may be that LBD, including DLB, shows a peripheral inflammatory phenotype consistent with a past history of chronic and/or recurrent infections, with a "burnt-out" or senescent adaptive immune response, which perhaps responds to further infection with impaired proliferation. Contrastingly, AD may be characterised by a relatively intact adaptive immune response, one which is not driven by infections but instead by chronic low-grade inflammatory conditions. In support of this theory, epidemiological data has shown DLB to be associated with increased frequency of infections [95] while chronic inflammatory conditions have been associated with AD [213], but not PD [313].

Another possible reason for the changes found in cells associated with adaptive immunity is that there could be a lower peripheral antigen load in DLB compared with AD. Patients with DLB appear to suffer with more trauma and infections than those with AD, and therefore one would assume that they would be subjected to higher rates of circulating antigens. One relevant factor may be the amounts of constituents of neuropathology present in the periphery. Markers of neuropathology for both DLB and AD are readily detected in body fluids such as blood and CSF, with promising scope for use as

diagnostic biomarkers [380, 381]. There may be differing levels of these markers in the blood of DLB and AD cases, accounting for the contrasting activation levels of the adaptive immune system. Alternatively, there may be unknown factors that have caused a disruption in the proliferation of activated adaptive immune system cells in response to antigen in DLB.

The only previous study examining PBMC in DLB did not examine cell populations, but specifically presented data regarding T cells stimulated with Aβ in AD and DLB [344]. The study found that only AD patients possessed a subset of T cells that were specifically activated by Aβ1-42, the major constituent of amyloid plaques. That there are circulating T cells specific to key markers of cerebral neuropathology in AD demonstrates a clear link between peripheral adaptive immunity and cerebral protein deposition. In support of this, in PD circulating T cells elicit a specific response to peptides derived from α-syn, the major component of LRP [382]. These findings in PD are supported by a genetic study showing that genetic variation in the *HLA* region is a risk factor for the development of PD [304], further supporting alterations in antigen presentation function and the adaptive immune system in PD.

### 3.3.3 Serum cytokine data

SILAD serum data showed significantly higher IL1β in DLB compared with AD and controls, and higher IL6 in DLB compared with controls. Notably, the serum concentrations of all cytokines investigated (apart from IL8) were higher in both dementia groups compared with the control groups, although in most cases these differences were not statistically significant. IL1β and IL6 have previously been shown to be linked. IL1β is a key, acute phase, pro-inflammatory protein that is known to be a potent inducer of IL6 [383]. Whilst it has been established that IL1β is a major pro-inflammatory cytokine, the function of IL6 is thought to be more subtle. IL6 has been identified as a pivotal cytokine in the transition from innate to adaptive immunity, playing a role in monocyte and T cell recruitment, and resolution of the acute inflammatory phase [384]. In addition, IL6 has been shown to be involved in T cell differentiation [385]. However, in animal models it has been shown that IL6 inhibits the production of IL1 and TNFα, suggesting an additional anti-inflammatory function [386, 387].

It is noteworthy that, after adjusting for the presence of infections in the preceding 6 weeks, serum IL1β remained significantly higher in DLB compared with AD but not with controls, and serum IL6 remained higher in DLB compared with controls. This supports the conclusion that the elevation of serum IL1β and IL6 in DLB is not driven by the systemic effects of recent infections.

The finding that these two cytokines are present at significantly higher concentrations in DLB compared with controls is novel, although a recent systematic review did identify numerous studies with similar increases in PD and PDD [336]. One of the two previous studies examining blood cytokine concentrations in DLB, published by King et al., found no alteration in any of the cytokines studied in SILAD, although IL6 was significantly higher than controls in a prodromal DLB group. More broadly, the study also found increased levels of IL1β, IL2, IL4 and IL10 in both MCI-AD and MCI-DLB compared with dementia and control groups [338]. The contrast in findings between SILAD and King et al. may be due to a number of factors. Whilst the DLB groups in each study appeared relatively well matched for age and gender, there were some notable differences. The DLB group in the King et al. study had a much shorter disease duration (approximately 2 years c.f. 4 years in SILAD), much higher UPDRS motor examination scores (43 c.f. 14.5 in SILAD) and higher frequency of participants taking anti-inflammatory drugs (38% c.f. 25% in SILAD). From these observations, it is possible that DLB participants in the King et al. study had a more PD-prominent symptom profile, and in addition their levels of peripheral cytokines may have been affected by higher use of anti-inflammatory drugs. These factors may explain the discordant findings, and may be secondary to local recruitment procedures leading to examination of different sub-populations of DLB phenotype.

The only other previous study in this area showed that serum IL6 was associated with worsening cognition in DLB, but it did not have a control group for comparison [337]. IL1β has been consistently been found to be elevated in PD, PDD and AD [235, 336], with the results from SILAD extending this pattern to DLB. It is also noteworthy that polymorphisms in the IL1β gene have been shown to be risk factors for the development of AD and PD [206, 302], but not yet in DLB, possibly as a result of limited DLB genetic data. There may be a

common and important role for IL1β in the pathogenesis of all of these neurodegenerative causes of dementia.

Interestingly, CSF IL6 has been shown to be significantly lower in DLB than in both AD and controls, with IL6 concentration additionally being found to be negatively correlated with MMSE score and positively correlated with α-syn CSF levels [341]. In contrast, CSF IL6 has also been found to be elevated in DLB compared with controls, albeit not significantly [342]. The role of serum IL6 in DLB still remains unclear, but the finding in SILAD of elevated serum IL6, along with previously reported elevation in prodromal DLB, suggests this cytokine may play a role specific to DLB through the entire process of prodromal pathogenesis to neurodegeneration. The functions of IL6 are complex and varied, with both pro- and anti-inflammatory properties, meaning that its role may indeed be nuanced and could change during the course of disease progression.

It is noteworthy that serum cytokine analysis in SILAD failed to reveal a significant difference in any cytokine concentration in AD compared to controls, a finding which contradicts many previous studies [235]. However it is worth mentioning, albeit with caution, that the median concentration of several cytokines were higher in the AD group compared with controls, but none reached significance. Post-hoc analysis was required as three groups were included in SILAD, which may have precluded the finding of significant differences between AD and controls. Furthermore, the spread of serum cytokine concentrations within the AD group was notably large and may have prevented group differences reaching statistical significance.

### 3.3.4 Stimulated cytokine data

Stimulated PBMC cytokine ratios did not show a significant difference between the three groups in SILAD. The purpose of these experiments was to examine the reactivity of PMBCs in response to different stimuli. LPS is a constituent of the cell wall of Gram-negative bacteria and is used to stimulate PBMC to assess cytokine production occurring from the presence of antigen. PHA is a known mitogenic agent that triggers mitotic activity of T cells but not B cells and is used to assess the tendency for T cell proliferation. In all three groups stimulation with LPS and PHA resulted in increased production of all cytokines

from PBMC compared with unstimulated supernatant. However, when comparing stimulated ratios between groups, no significant differences were discovered. The lack of difference in cytokine production post-stimulation indicates that peripheral immune cells in DLB and AD may show an unchanged level of sensitivity to *ex-vivo* immune challenges related to T cell proliferation or antigen presentation.

### 3.3.5 Correlation with clinical data

Overall, no significant associations were found between markers of peripheral inflammation and the clinical features of DLB. This is contrary to one previous paper that found a significant association between serum TNFα and neuropsychiatric symptoms in DLB [337], while CSF IL6 has been shown to negatively correlate with MMSE score. Furthermore, severity of Parkinsonism and cognitive impairment have previously been shown to be positively correlated with serum IL6 and TNFα, and negatively correlated with IL1β, IL2 and IL4, albeit in a pooled DLB and MCI-DLB group [338].

The lack of correlation with clinical data in SILAD could be explained by the absence of a mechanistic link between peripheral inflammation and DLB symptomatology. As previously discussed, the symptom profile in DLB includes fluctuations and lends itself to being caused by synaptic dysfunction, which may not be associated with changes in peripheral inflammation. It should be noted that the findings published previously in DLB have not been consistent and there is no clear or overarching mechanism that has been proposed in this field. Alternatively, significant correlations may not have been discovered in SILAD due to a type II error, possibly related to a range of possible methodological factors, as discussed further in chapter 3.3.7.

### 3.3.6 Effect of *APOE* genotype

When control and DLB participants were grouped by *APOE* genotype, no differences were found in stimulated cytokine ratios. Contrastingly, in AD several stimulated cytokine ratios were significantly lower in *APOE* ε4 carriers compared with non-carriers, including for IL1β, IL4, IL6 and TNFα. This

supports the hypothesis that the tendency of PBMC to produce cytokines is modulated by *APOE* genotype in AD, but not in DLB or controls.

This finding implies that *APOE* may be associated with down-regulating cytokine production in AD. It is known that possession of at least one *APOE* ε4 allele significantly increases the risk of developing AD [105], but it is not associated with quicker progression once an individual already has AD [388]. The interplay between *APOE* and peripheral cytokines remains unclear, and may indeed vary according to the stage of disease.

### 3.3.7 Strengths and limitations

This clinical study is one of only a handful that have examined peripheral inflammation in DLB. The use of flow cytometry to investigate peripheral immune cell populations in DLB is unique, with novel results identified.

Recruitment to the 120 participant target was not possible due to challenges in obtaining appropriate referrals and because of resource limitations. DLB is known to be underdiagnosed and during recruitment it appeared that some people had indeed been previously misdiagnosed, particularly with AD. This sometimes contributed to delayed recruitment as patients were re-evaluated, sometimes with further brain imaging, to help to confirm the diagnosis of DLB. However, a large number of patients were recruited to the study and it was still powered to detect significant differences between groups.

The cross-sectional nature of any clinical study does raise some limitations. Examination of peripheral inflammatory markers was performed at just one point in SILAD, negating the ability to infer information about the interaction between disease progression and inflammation. The inclusion of an MCI-DLB group may have proven useful in assessing the temporal effect of disease progression on peripheral inflammation. Correlations were performed with clinical details including duration of disease, but the onset of symptoms as a clinical measure is unreliable and of limited significance. Furthermore, details such as fluctuation in cognition were measured using a rating scale at the single study visit rather than assessment over repeated visits, although fluctuation in particular is a particularly difficult variable to quantify even over a longer period of time. Consideration had to be given in the planning phase of

the study as to the feasibility of performing follow-up visits and blood analysis with limited resources.

A related potential limitation to the study design concerns the exclusion of severe DLB cases, as measured by low MoCA scores or implied through an inability to consent to participate. This may have limited the range of DLB cases investigated and further diminishes the ability to assess a temporal relationship between inflammation and disease stage. However, recruitment of such patients has particular challenges with regards to ethical research practice.

A variety of different methods have been used in previous studies to examine serum cytokine levels, including ELISA, cytometric bead array, MSD and Luminex. The assessment of cytokine levels in clinical research is known to pose a number of challenges. Zhou et al. has highlighted the critical importance of standardising methodological factors when assessing cytokine levels, including consistency in phlebotomy timing to reduce the effect of diurnal variation, and also ensuring serum samples are processed under the same conditions [389]. Of particular concern is the degradation of some cytokines, and the generation of others, after phlebotomy. This is a particular problem 2-4 hours after phlebotomy and even following long-term freezer storage. Another group has recommended that serum samples be frozen as soon as possible after phlebotomy, preferably within 1 hour, and that they should be frozen at -80ºC for a maximum of two years without any freeze-thaw cycles to prevent degradation of cytokines [390]. Failure to ensure that samples are processed consistently could bias study results. It should be noted that these factors were mitigated in the protocol for SILAD, with bloods taken within set times of the day where possible and the conditions of blood processing being consistent.

The propensity of PBMC to produce cytokines in response to stimuli *ex-vivo* does particularly depend on experimental conditions, including the concentration of stimuli used. The concentrations of stimuli used in SILAD were consistent with some previous literature, but notably different to other previous work. For example, Reale et al. used an LPS concentration of 1ng/ml [356] – 1000 times more diluted than the concentration used in SILAD. The only other difference in experimental conditions was the use of Mesoscale in

SILAD to quantify cytokine concentrations, compared with the ELISA kit used in the Reale et al. study. Even when using similar research methods and diagnostic criteria, there still appears to be a wide variation in results between studies examining blood and CSF cytokine concentrations.

Finally, the lack of detection of differences in stimulated cytokine ratios between controls, AD and DLB could be due to a limitation in methodology. The whole PBMC population was stimulated for each case, with supernatant then analysed for cytokine concentration. An obvious limitation is that this method does not allow examination of the individual responses of each PBMC subset to stimulus. For example, it is plausible that simulated B cells could have produced significantly more of a specific cytokine, but that stimulated helper T cells could have produced the reverse, meaning that no overall difference would have been detected when the supernatant was evaluated. In order to examine these changes with more sensitivity, cell sorting using flow cytometry would need to be utilised in order to examine each subset of PBMC separately. This would necessitate a larger number of PBMC to study for each case, requiring a larger draw of blood from each study participant.

### 3.3.8 Summary

In summary, data from SILAD has shown increased levels of serum IL1$\beta$ and IL6 in DLB. The increase in concentration of these cytokines in DLB is consistent with much of the previous literature examining serum cytokines AD, PD and PDD. This adds weight to the hypothesis that increased pro-inflammatory systemic cytokines may be key players in the pathogenesis of a number of neurodegenerative disorders.

Elevation of these pro-inflammatory cytokines that are typically produced in an innate immune response would normally lead to activation of the adaptive immune system, with increased helper T cells and activated B cells, followed by negative feedback to avoid overproduction of inflammatory cytokines during an innate immune response [391]. However, in DLB (when compared with AD) the proportion of helper T cells and level of B cell activation were in fact decreased, indicating reduced activity of the adaptive immune system. The decrease in these two adaptive immune system cell subsets in DLB supports a profile of immunosenescence, perhaps driven by chronic antigen stimulation

due to repeated infections. This “burnt-out” profile of the adaptive immune system in DLB may lead to preferential production of pro-inflammatory cytokines in response to further immune stimulation, rather than cell proliferation and differentiation, encouraging chronically raised serum IL1β and IL6 in DLB.

There are two further possible explanations for the reduction in helper T cell populations in DLB compared with AD. One reason is that there could be a downregulated helper T cell response to antigen in DLB. Alternatively, there could be less chronic antigen load in DLB. It is difficult to ascertain which of these scenarios is most accurate due to limitations in the methodology of the flow cytometry panel used in SILAD, meaning that further work is certainly required in order to establish the precise phenotype of these cells in DLB.

The results of this study have revealed original findings relating to the peripheral immune phenotype in DLB. The following chapter will present the details of studies examining cerebral immunophenotype in DLB and AD.

# Chapter 4: Cerebral inflammation in DLB and AD

Small cohort sizes, conflicting results and a limited number of studies have curbed our understanding of the role of cerebral inflammation in DLB to date. Previous work in AD and PD has supported an alteration in microglial activity in these diseases, with microglial activation known to correlate with cognitive decline in AD. There is, however, a lack of consensus on the role of cerebral inflammation in DLB.

In this study, cerebral inflammation in DLB was investigated using immunohistochemistry to examine microglial phenotype in post-mortem human brain tissue of DLB and control cases. An AD group was not included in this study as microglial immunophenotype in AD had already been examined by the Boche group in Southampton (publication in preparation), with a summary of results presented in section 4.2.12. The study presented in this chapter was entitled CIDL (**C**erebral **I**nflammation in **D**ementia with **L**ewy bodies); the methodology and results of which are presented below.

## 4.1 Methods

### 4.1.1 Power calculations

A medical statistician, Mr Scott Harris, was consulted in the design of CIDL, including in generating power calculations.

Due to resource constraints and in order to be sufficiently powered, it was decided that one neuroanatomical region would be examined from a large number of cases rather than a large number of regions in fewer cases. Studies examining microglial activation in post-mortem brain tissue typically include 5-30 cases per group. A sample size of 30 in each group, with a standard 80% power and 5% significance would be able to detect standardised effect sizes of

at least 0.516. From previous studies we have shown a range of standardised effect sizes from 0.65 (difference of 20.6 with s.d. 31.6) to 1.8 (difference of 0.18 with s.d. 0.1), so the study should have been adequately powered to pick up reasonable effect sizes.

### 4.1.2 Ethics

Post-mortem human brain tissue was sourced from 30 DLB cases and 29 non-neurological control cases from the Medical Research Council (MRC) London Neurodegenerative Disease Brain Bank (LNDBB) and the South West Dementia Brain Bank (SWDBB). Each brain bank provided blanket ethical approval for accepted studies – MRC LNDBB reference 08/MRE09/38+5 from NREC Wales and SWDBB reference 08/H0106/28+5 from NREC South West Central Bristol. This study, CIDL, was registered with the University of Southampton Ethics and Research Governance Online (ERGO ref: 13170) prior to commencement. All methods, experiments and reagents were risk assessed in line with University of Southampton Health and Safety regulations.

### 4.1.3 Case selection

I selected eligible DLB cases with assistance from Prof Boche, based on neuropathology reports following full post-mortem examination. Clinical details were also reviewed by me for each case to ensure the clinical symptoms prior to death were consistent with a diagnosis of probable DLB. SWDBB provided electronic details for all cases, while LNDBB allowed a physical visit to their site to review paper records.

Inclusion criteria for selection into the DLB group are shown below in table 4.1. It should be noted that cases were excluded if the post-mortem delay was greater than 72 hour and if there was severe co-existing vascular disease, cerebral amyloid angiopathy, or any disease or insult leading to immune activation. Cases were also excluded if the Braak ptau stage was rated as greater than 3, indicating presence of significant pathology that could represent a co-existing neuropathological diagnosis of AD [392]. The purpose of these criteria were to minimise any potential bias caused by co-existing pathology.

**Table 4.1: Inclusion criteria for DLB group in CIDL**

| |
|---|
| Diagnosis of DLB made on post-mortem report |
| Aged 60 or older at death |
| Post-mortem delay less than 72 hours |
| No co-existing severe vascular disease or cerebral amyloid angiopathy |
| Braak ptau stage less than or equal to 3 |

Control cases were also selected by me after review of neuropathological post-mortem reports. Controls were matched for gender, post-mortem delay and age at death. Inclusion criteria for selection into the control group were similar to the DLB group and are shown below in table 4.2.

**Table 4.2: Inclusion criteria for control group in CIDL**

| |
|---|
| Neuropathologically normal on post-mortem report |
| Aged 60 or older at death |
| Post-mortem delay less than 72 hours |
| No co-existing severe vascular disease or cerebral amyloid angiopathy |
| Braak ptau stage less than or equal to 3 |

Brain banks were asked to provide the following details for all cases: age at death, gender, post-mortem delay, *APOE* genotype and disease duration. Tables 4.3 and 4.4 provide lists of characteristics for control and DLB cases selected in CIDL. Analysis of baseline characteristics is presented in the results section (4.2.1).

**Table 4.3: Details of control cases used in CIDL**

| Case ID | Gender | Age (years) | PM delay (hours) | Braak ptau stage | *APOE* genotype |
|---|---|---|---|---|---|
| A359/08 | F | 80 | 3 | 1 | 3,3 |
| A407/13 | F | 80 | 22 | 2 | 3,4 |
| A310/09 | F | 84 | 35 | 2 | N/A |
| A133/12 | F | 88 | 39 | 3 | 3,3 |
| A261/12 | M | 63 | 23 | 0 | 3,3 |
| A388/12 | M | 65 | 26 | 1 | 3,3 |
| A273/12 | M | 67 | 25 | 1 | 3,3 |
| A053/11 | M | 77 | 11 | 0 | 3,3 |
| A127/11 | M | 73 | 23 | 0 | 3,3 |
| A213/12 | M | 78 | 24 | 3 | 3,3 |
| A265/08 | M | 79 | 47 | 2 | 2,3 |
| A114/12 | M | 82 | 24 | 2 | 3,3 |
| A033/11 | M | 82 | 47 | 1 | 3,4 |
| A134/00 | M | 86 | 6 | N/A | 3,3 |
| A002/13 | M | 90 | 45 | 0 | 2,3 |
| A049/03 | M | 79 | 24 | 0 | 2,3 |
| A346/10 | F | 84 | 34 | 2 | 2,3 |
| A130/12 | F | 89 | 43 | 2 | 3,3 |
| A051/14 | F | 76 | 22 | 2 | N/A |
| 786 | M | 85 | 30.5 | 2 | 3,3 |
| 803 | M | 77 | 42 | 1 | 3,3 |
| 818 | F | 87 | 47 | 3 | 2,3 |
| 851 | F | 68 | 38.75 | 0 | 2,3 |
| 870 | F | 90 | 41 | 2 | 3,3 |
| 877 | M | 82 | 67 | 2 | 3,3 |
| 894 | M | 74 | 57.5 | 0 | 3,3 |
| 887 | F | 74 | 39.5 | 1 | 3,3 |
| 940 | M | 94 | 64.25 | 2 | 2,4 |
| 943 | F | 70 | 33.25 | 2 | 3,3 |

**Table 4.4: Details of DLB cases used in CIDL**

| Case ID | Gender | Age (years) | PM delay (hours) | Braak ptau stage | Disease duration (years) | *APOE* genotype |
|---|---|---|---|---|---|---|
| A225/03 | M | 70 | 8 | 1 | 6 | N/A |
| A056/01 | F | 80 | 17 | 0 | 16 | N/A |
| A040/10 | F | 87 | 9 | 2 | 2 | 3,3 |
| A109/01 | M | 65 | 5 | 0 | 11 | N/A |
| A231/09 | M | 66 | 35 | 2 | 7 | N/A |
| A242/04 | M | 72 | 22.45 | 1 | 9 | N/A |
| A175/09 | M | 73 | 5.75 | 1 | 9 | N/A |
| A204/07 | M | 74 | 18 | 2 | 12 | 3,3 |
| A036/10 | M | 81 | 24 | 3 | 5 | N/A |
| A028/10 | M | 81 | 57 | 3 | 7 | 3,3 |
| A241/11 | M | 82 | 32 | 1 | 8 | N/A |
| A190/03 | M | 83 | 38 | 3 | 6 | N/A |
| A273/05 | M | 86 | 8 | 0 | 5 | 3,3 |
| A245/09 | M | 78 | 46 | 3 | 4 | N/A |
| A263/05 | M | 78 | 41 | 2 | 8 | N/A |
| A273/07 | M | 71 | 19 | 2 | 10 | N/A |
| A304/06 | F | 92 | 55 | 3 | 9 | N/A |
| A025/98 | M | 75 | 24 | N/A | 5 | N/A |
| A076/99 | M | 59 | 30 | N/A | 4 | N/A |
| A339/96 | M | 86 | 48 | N/A | 2 | N/A |
| 701 | M | 73 | 8 | 3 | 7 | 3,4 |
| 738 | F | 76 | 33 | 3 | 13 | 3,3 |
| 743 | M | 86 | 15.25 | 3 | N/A | 3,3 |
| 756 | M | 69 | 38 | 0 | 5 | 3,3 |
| 776 | F | 79 | 26 | 2 | 1 | 3,3 |
| 817 | M | 89 | 40 | 2 | 1 | 3,4 |
| 823 | M | 80 | 38 | 2 | 4 | 3.4 |
| 832 | M | 76 | 26 | 0 | 7 | 3,3 |
| 846 | F | 67 | 20 | 0 | 5 | 3,3 |
| 901 | M | 94 | 32.25 | 3 | N/A | 3,3 |

### 4.1.4 Immunohistochemistry

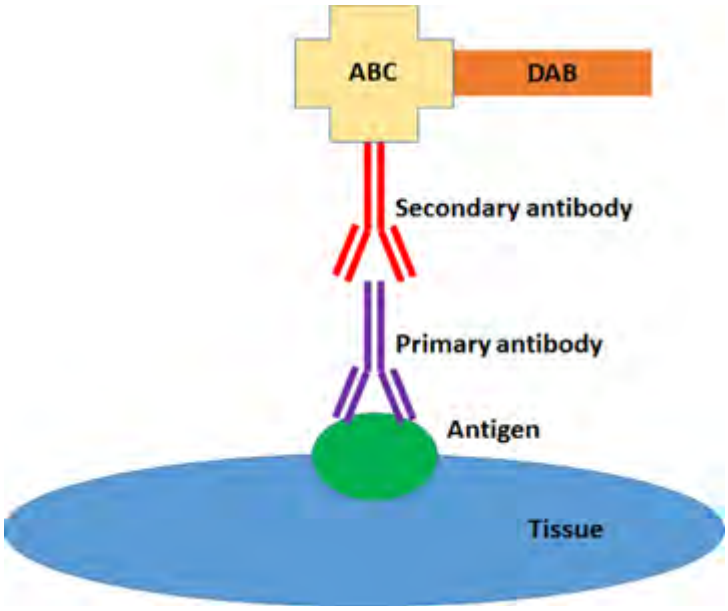
Brain banks provided formalin-fixed and paraffin-embedded (FFPE) mounted sections of the superior temporal sulcus (from BA 21) at 4μm thickness.

All immunohistochemistry experiments were performed in the Histochemistry Research Unit (HRU) at the University of Southampton. Experiments were typically split into two runs due to the number of slides involved. Cases from the two brain banks (LNDBB and SWDBB), and the two groups (DLB and controls), were equally split between each run to ensure comparability of immunolabelling. Positive controls containing the protein of interest, typically myeloid tissue (e.g. tonsil), were used in every run to confirm that the experiment had been successfully conducted.

I conducted or supervised the majority of experiments. Immunostaining for Aβ, ptau and Iba1 was performed by Jenny Norman in the HRU. Immunostaining for α-syn was performed by the Cellular Pathology Department, University Hospitals Southampton. Undergraduate medical students performed some of the immunohistochemistry experiments under my supervision.

The full protocol used for immunohistochemistry experiments, along with a list of reagents used, is available in Appendix C. A brief summary of the protocol follows, along with a schematic in figure 4.1. FFPE brain tissue was rehydrated through graded alcohol solutions and water. After each subsequent step all slides were rinsed with trisaminomethane buffered saline (TBS). Endogenous peroxidase was blocked with a 3% hydrogen peroxide solution. An antigen retrieval method was then employed, which involved heating the tissue slides whilst soaked in a buffer. Blocking medium was used to reduce non-specific staining. The primary antibody (which targeted the antigen of interest) was then applied and slides incubated either for 90 minutes or overnight. This was then followed by incubation with a secondary antibody to allow treatment of tissue with avidin-biotin complex (ABC) to amplify the staining signal. This complex was then detected by 3,3'-diaminobenzidine (DAB) which produced a brown precipitate. Tissue was counterstained with haematoxylin to stain for general parenchymal structure, enabling localisation of the staining and improved differentiation of grey and white matter. Dehydration of slides was then performed before the slides were mounted and left to dry.

**Figure 4.1: Schematic of immunohistochemistry experiment**




### 4.1.5 Primary antibodies used in immunohistochemistry

All antibodies were subject to experiments to titrate concentration and optimise antigen retrieval methods. The majority of antibodies had already been optimised by the Boche laboratory prior to the commencement of this study. Further details of all primary antibodies used in CIDL are provided below, along with experimental conditions in table 4.5.

Three neuropathology markers were selected, details of which are listed below.

- **α-syn** is a protein known to be the primary constituent of LRP. Mouse anti-human clone KM51 from Novocastra was selected as it is the antibody of choice of the Cellular Pathology Department, University Hospitals Southampton. This particular antibody has been shown to produce minimal background or "synaptophysin-like" immunoreactivity compared with other α-syn antibodies [393].
- **Pan-Aβ** is a marker of amyloid plaques, one of the key neuropathological markers of AD. Mouse anti-human clone 4G8 from Covance-Biolegend was selected as it has been previously optimised in the Boche laboratory.
- **ptau** is a marker of neurofibrillary tangles, one of the other key neuropathological markers of AD. Mouse anti-human clone AT8 was selected as it has been previously optimised in the Boche laboratory.

Three markers were chosen to identify microglial phenotype, as listed below.

- **Iba1** is a widely used and specific marker of microglia [169]. It allows for excellent staining of all microglia, whether resting, activated, phagocytic or dystrophic [166, 171]. It has been shown to be upregulated upon microglial activation [394] and has been identified as a marker of microglial motility due to its involvement in actin cross-linking, needed for cell migration [174].
- **HLA-DR** is expressed on the surface of antigen-presenting cells and is the human analogue of the Major Histocompatibility Complex class II antigen presentation complex. It is widely used as a marker of activated microglia [166, 169, 172] and was chosen to assess the antigen presentation phenotype of microglia.
- **CD68** is a marker known to localise to the lysosomal membrane in microglia and perivascular macrophages [166, 169], indicating phagocytic activity. It is also known as macrosialin in mice.

FcγR are cell surface receptors expressed on microglia that bind to the constant domain of IgG. Activation of FcγR causes release of pro-inflammatory cytokines and controls the extent of phagocytosis [166]. Human microglia express these receptors at very low concentrations normally, but levels of the activating FcγR are known to be increased in AD, especially around Aβ plaques [230]. The four known human FcγR markers are listed below and were included to measure the level of antibody-dependent phagocytic activity of microglia.

- **CD64** (FcγRI) - activating, high affinity to IgG
- **CD32a** (FcγRIIa) - activating, low affinity to IgG
- **CD32b** (FcγRIIb) - inhibitory, low affinity to IgG
- **CD16** (FcγRIIII) - activating, low affinity to IgG

Lastly, two markers against anti-inflammatory proteins were chosen.

- **IL4R** is a cell surface receptor for the anti-inflammatory cytokine IL4, which is known to induce alternative activation of microglia [395].
- **CHI3L1** is a cell surface receptor marker of alternative activation of microglia [395] and a potential biomarker in AD [263, 264].

**Table 4.5: Details of primary antibodies used in immunohistochemistry**

| Primary antibody | Species | Clone | Manufacturer | Concentration | Pre-treatment method |
|---|---|---|---|---|---|
| α-synuclein | Mouse | KM51 | Novocastra | 1:100 | Performed by Research Histology department |
| β-amyloid | Mouse | 4G8 | Covance-Biolegend | 1:2000 | 80% formic acid |
| Ptau | Mouse | AT8 | Thermoscientific | 1:500 | Citrate buffer in pressure cooker |
| Iba1 | Rabbit | 019-19741 | Wako/Alpha-labs UK | 1:750 | Citrate buffer in pressure cooker |
| HLA-DR | Mouse | M0755 | Dako | 1:200 | Citrate buffer in microwave |
| CD68 | Mouse | M0876 | Dako | 1:50 | Citrate buffer in microwave |
| CD64 | Goat | AF1257 | R&D systems | 1:100 | EDTA buffer in microwave |
| CD32a | Mouse | 13D7 | Abcam | 1:2000 | EDTA buffer in pressure cooker |
| CD32b | Rabbit | EP888Y | Abcam | 1:4000 | EDTA buffer in microwave |
| CD16 | Goat | AF1597 | R&D systems | 1:150 | EDTA buffer in microwave |
| IL4R | Rabbit | HPA050124 | Sigma | 1:100 | EDTA buffer in pressure cooker |
| CHI3L1 | Goat | AF2599 | R&D systems | 1:100 | Citrate buffer in pressure cooker |

### 4.1.6 Image capture

All slides were physically marked by an experienced neuropathologist (Prof James Nicoll, University of Southampton) for identification of the superior temporal sulcus of the middle temporal gyrus (brain region BA21). This allowed for accurate and consistent sampling of the area of interest.

The same method of image capture was used for all immunolabelled tissue. Images were captured using a Dotslide hardware digital camera (Olympus, Hamburg), which was attached to a light microscope. The same hardware model and camera settings were used for all sections in a given antibody batch to ensure consistency of the optical properties of images and to allow for comparison in analysis. All images were taken at x20 magnification. Image capture was performed blind to group status and by either me, or undergraduate medical students under my supervision.

Using the Tissue Micro-Array module on Dotslide, 30 pictures were taken of both grey and white matter around the region of interest. Each picture box was $0.5mm^2$ in size, meaning the total area sampled per slide was $15mm^2$. Grey matter was sampled using a zig-zag pattern of images in order to ensure all six cortical areas were sampled (Figure 4.2). White matter was sampled using a similar method and in the same region of interest as the grey matter had been sampled.

There were a number of circumstances where the methods above did not apply. Firstly, digital images were not taken of $\alpha$-syn immunostaining. Due to the sparse and dispersed pattern of LRP it was decided that the image capture technique described above would not accurately reflect the severity of LRP pathology. Secondly, since there was no plaque or tangle pathology present in the white matter, images were not taken of the white matter for tissue immunostained against A$\beta$ and ptau. Lastly, there was no IL4R immunostaining visible in the white matter of any case and therefore it was decided not to take images of the white matter for this marker.

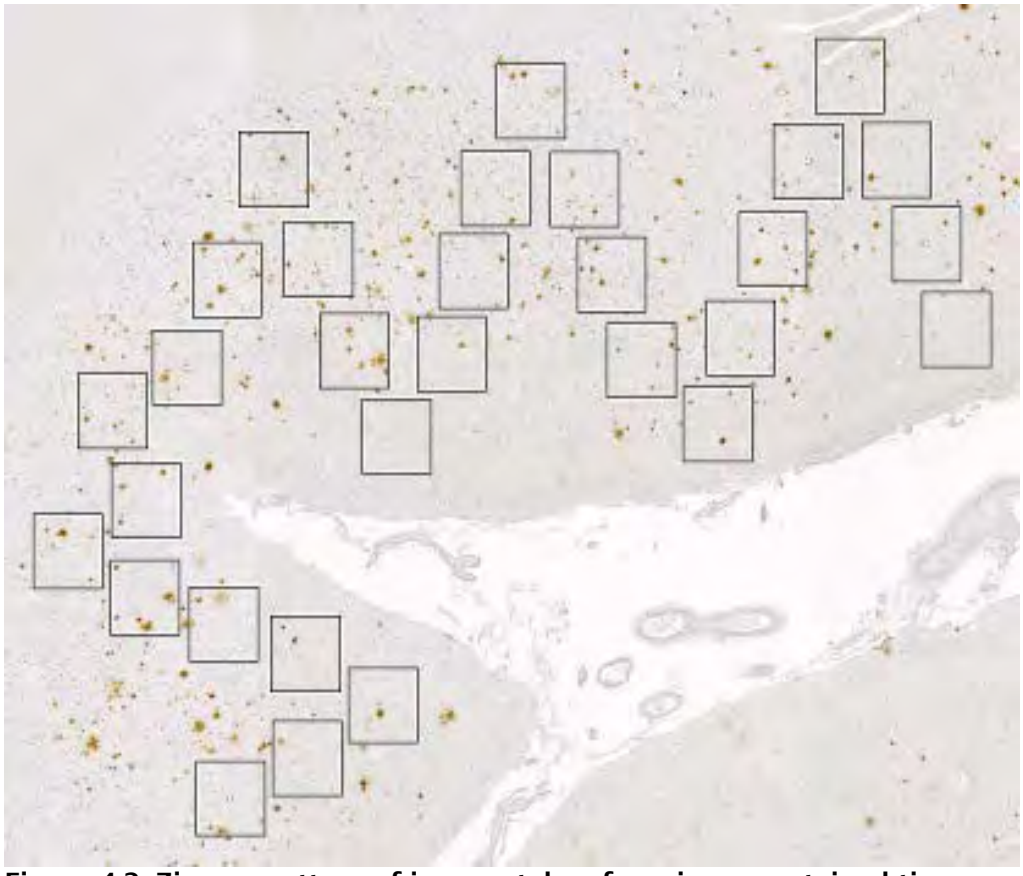


**Figure 4.2: Zig-zag pattern of images taken from immunostained tissue**

### 4.1.7 Quantification

As previously described, image capture was not performed for quantification of LRP. This was instead quantified using a semi-quantitative analysis, as recommended by the international consensus criteria for neuropathological diagnosis of DLB [9]. Advice and training was sought from an experienced neuropathologist (Prof James Nicoll). The same region of interest that was used for image capture was assessed directly using a light microscope (Nikon Eclipse 50i) using low magnification (x4). A score of 0-4 was attributed to each case, according to the criteria listed below.

- 0 = None
- 1 = Mild (sparse LB or LNs)
- 2 = Moderate (more than one LB in a low power field and sparse LN)
- 3 = Severe (four or more LB and scattered LN in a low power field)
- 4 = Very severe (numerous LB and numerous LN)

For all other markers, images of immunostained tissue were quantified using ImageJ software (http://rsbweb.nih.gov/ij/). ImageJ is an open-access, Java-based, imaging processing programme developed at the National Institutes of Health (USA). It can used to display, edit, analyse and process digital images. ImageJ was used to optimise the process of quantification of immunostained tissue using a number of different parameters, which were then reviewed and agreed with Prof Boche. The desired outcome was to enable ImageJ to accurately quantify the level of immunostaining present. This included ensuring that both non-specific background staining was excluded from quantification, but also that any specific staining present was not overlooked. Images processed during optimisation were compared, side-by-side, with original digital images to confirm the accuracy of image processing.

ImageJ was used to develop macros, based on the algorithms agreed during optimisation above. The macro typically included the following steps. Blue colour was removed from the images using a red-green-blue (RGB) recolour plugin and then images were converted to 8-bit black and white. A threshold for detection was then set to allow ImageJ to quantify the extent of staining present in each picture. The same macro, and threshold, was then used for each picture and case for a marker, ensuring consistency. An example of a macro written for Image J is shown below in Figure 4.3. This process allowed for ImageJ to produce a percentage value indicating the extent of immunostaining in the area of the picture, expressed as “percentage protein load”. Results from the 30 pictures per case were copied from ImageJ into a Microsoft Excel spreadsheet and plotted as a scatter plot to check for outliers. The processed images of outliers were verified in order to confirm their accuracy and excluded from analysis if necessary.

During optimisation of image processing it was deemed to be beneficial to change certain ImageJ processing steps for some of the markers. For images of tissue stained for Aβ, ptau, Iba1 and CHI3L1 the “subtract background” step was not required, and for CD32b an “enhance contrast” step was added before the RGB recolour step. These modifications enabled more accurate representation of the extent of immunostaining and were consistently applied to all images for that particular marker. The ImageJ parameters used, including the threshold used for each marker, are shown in table 4.6.

```
run("Subtract Background...", "rolling=50 light");
run("Enhance Contrast...", "saturated=0.6");
run("RGB Recolor", "red=0 red=0 green=0 green=0 blue=1 blue=0");
run("8-bit");
setAutoThreshold("Default");
setThreshold(0, 0);
run("Convert to Mask");
run("Set Measurements...", "  area_fraction redirect=None decimal=3");
run("Measure");
for (i=0;i<=1000000;i++)
```

**Figure 4.3: Image J macro**

**Table 4.6: List of parameters used during ImageJ processing**

| Marker | Subtract background | Enhance contrast | Threshold (grey matter) | Threshold (white matter) |
|---|---|---|---|---|
| **Aβ** | No | No | 36 | N/A |
| **Ptau** | No | No | 35 | N/A |
| **Iba1** | No | No | 32 | 32 |
| **HLA-DR** | Yes | No | 55 | 50 |
| **CD68** | Yes | No | 47 | 45 |
| **CD64** | Yes | No | 50 | 50 |
| **CD32a** | Yes | No | 60 | 52 |
| **CD32b** | Yes | Yes | 0 | 0 |
| **CD16** | Yes | No | 52 | 50 |
| **IL4R** | Yes | No | 52 | N/A |
| **CHI3L1** | Yes | No | 68 | 61 |

Quantification was performed blind to group status. The process of image manipulation described above is illustrated in Figure 4.4.

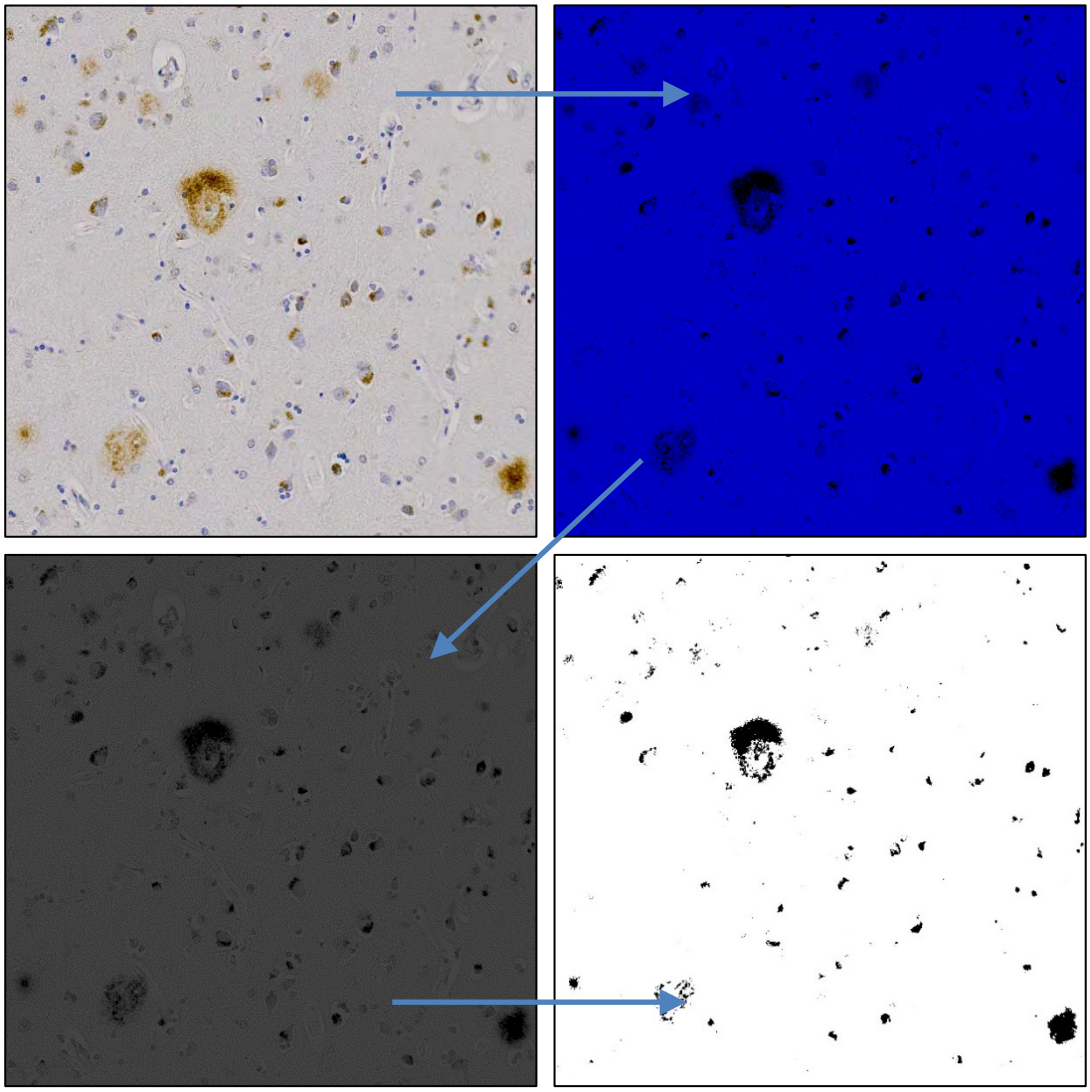


**Figure 4.4: ImageJ processing of digital images**
Pictures showing the processing of an original image from a slide immunostained for Aβ (top left) at x20 magnification. RGB recolour was used to remove all red and green from the image (top right). The image is then converted to an 8-bit greyscale image (bottom left). The setting of a threshold for detection of the area of dark staining results in a black and white image (bottom right). The stained area in black is then divided by the total area to produce a percent protein load.

### 4.1.8 Data analysis

All data were entered into SPSS statistical software for analysis (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp). Results were recorded as percentage (%) protein load for each digital image captured of the immunostained tissue. Following consultation with medical statistician Mr Scott Harris (University of Southampton), the mean value of the 30 pictures taken in each case (for both grey and white matter separately) was used to represent the overall protein load of that particular marker in each case.

For every marker, the mean protein load from each case was assessed for normality using histograms and QQ plots. With all markers, the data was deemed to be non-normal in distribution. Therefore the Mann-Whitney U test was used to compare protein load between the DLB and control groups in all assessments. Two-tailed P values of less than 0.05 were deemed to be significant.

For analysis of correlations between markers, or between grey and white matter for each marker, the non-parametric Spearman Rank test was used to test for significant associations. Two-tailed P values of less than 0.01 were deemed to be significant to allow for testing of multiple correlations.

Graphs were then prepared using GraphPad Prism (Version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

## 4.2 Results

30 DLB and 29 control cases were selected to be included in CIDL. Details of all cases are shown in the methods section in tables 4.3 and 4.4. Analysis of baseline characteristics is presented below, followed by data showing protein loads of neuropathological and inflammatory markers. Protein load of inflammatory markers were also tested for association between grey matter and white matter. Lastly, data were analysed for correlations between markers of inflammation and markers of neuropathology.

### 4.2.1 Baseline characteristics

Assessment of the baseline characteristics of cases was performed. Table 4.7 shows a summary of data in each group for age at death, gender, post-mortem delay, Braak ptau stage, disease duration and *APOE* genotype.

**Table 4.7: Baseline characteristics for CIDL**

| | Controls (n=29) | DLB (n=30) |
|---|---|---|
| Age (years) | 79.4±8.0 | 77.6±8.3 |
| Gender (M:F) | 17:12 | 24:6 |
| Post-mortem delay (hours) | 33.9±15.5 | 27.3±14.6 |
| Disease duration (years) | - | 6.7±3.6 |
| Braak ptau stage | 1.4±1.0 | 1.7±1.2 |
| *APOE* genotype ≥1 ε4 allele | 3/27 | 3/14 |

Data presented as mean ± standard deviation

Age at death was deemed normally distributed following analysis of QQ plots and Shapiro-Wilk tests. There was no statistically significant difference in age between the control and DLB groups as determined by Independent samples T-test (controls mean 79.4 years [sd. 8.0] c.f. DLB mean 77.6 years [sd. 8.3], P=0.397). There was no missing data for this variable.

Despite there being proportionately more males than females in the DLB group compared with the control group, the difference in gender between groups was

not statistically significant, as determined by Pearson Chi-squared test ($\chi^2(1)=3.179$, P=0.075). There was no missing data for this variable.

Post-mortem delay was deemed normally distributed following analysis of QQ plots and Shapiro-Wilk tests. There was no significant difference in post-mortem delay between groups, as determined by Independent samples T-test (controls mean 33.9 hours [sd. 15.5] c.f. DLB mean 27.3 hours [sd. 14.6], P=0.096). There was no missing data for this variable.

The ptau Braak stage variable was deemed to be ordinal in nature. Utilising a 2x4 contingency table, there was no significant difference in ptau Braak stage between groups, as determined by Pearson Chi-squared test ($\chi^2(3)=4.360$, P=0.235). There was missing data for 1 control case and 3 DLB cases.

Analysis of differences between groups in *APOE* genotype was hampered by limited data received from the brain banks, particularly for the DLB cases. Three control cases and three DLB cases were positive for one or more *APOE* $\varepsilon$4 alleles. There was no significant difference in *APOE* genotype between groups, as determined by Fisher's Exact Test (P=0.393). There was missing data for 2 control cases and 16 DLB cases.

Duration of disease for DLB was deemed to be normally distribution following analysis of QQ plots and Shapiro-Wilk tests. The mean duration of disease for DLB cases was 6.7 years. There was missing data for 2 DLB cases.

### 4.2.2 Localisation of immunostaining

All immunostained slides were assessed for localisation of staining, with advice provided by an experienced neuropathologist (Prof James Nicoll). Table 4.8 describes the localisation of staining for all neuropathological and inflammatory markers examined in CIDL.

Example images taken of brain tissue immunostained for all markers are shown within each of the following sub-sections. All example images presented were selected from DLB cases.

**Table 4.8: Summary of localisation of immunostaining**

| Marker | Description of localisation of immunostaining |
|---|---|
| **α-syn** | LB localised to deep cortical layers. Variable level of fine, granular staining suggesting background or synaptic immunoreactivity. |
| **Aβ** | Plaques with a range of appearances, from diffuse plaques to those with dense cores. Present in all cortical layers. Staining in blood vessel walls representing cerebral amyloid angiopathy. |
| **Ptau** | Minimal staining in majority of cases. Some DLB cases had scattered neuronal cell body staining, intracellular tangles and neuropil threads. Evidence of some clustering of dystrophic neurites, presumably around plaques. |
| **Iba1** | Extensive staining of microglial cell bodies and processes. Range of morphological features visible, including some cells with swollen cell bodies and short processes, some with a ramified appearance and others with beaded processes. Staining of perivascular macrophages. Similar staining in white matter. |
| **HLA-DR** | Staining of microglial cell bodies and processes, but to a lesser extent compared with Iba1. Strong staining of perivascular macrophages. Similar staining in white matter. |
| **CD68** | Intracellular cytoplasmic staining of a subpopulation of microglial cells, consistent with localisation to microglial lysosomes. Evidence of clustering, presumably around plaques. Some staining of perivascular macrophages. Similar staining in white matter. |
| **CD64** | Extensive staining of a large number of microglial cell bodies and processes, along with perivascular macrophages. More extensive than Iba1 or HLA-DR staining. Similar staining in white matter. |
| **CD32a** | Widespread, but less well-defined, staining of microglial processes, and some staining perivascular macrophages. Similar staining in white matter. |
| **CD32b** | Neuronal nuclear staining. No obvious microglial localisation. No white matter staining. |
| **CD16** | Faint staining of some microglial cells. Localisation to small circular cells within vessel lumens, presumably monocytes or natural killer cells. |
| **IL4R** | Mixture of staining of star-shaped cells presumed to be astrocytes, along with extensive astrocytic end-feet processes on the pial surface (termed glia limitans). Staining appeared to cluster, possibly around plaques. |
| **CHI3L1** | Staining of neuronal cytoplasm, with some microglial staining. |

### 4.2.3 Summary of data in grey matter

Protein loads of all neuropathological and inflammatory markers were deemed to be non-normal in distribution following review of QQ plots. Therefore, the Mann-Whitney U test was used to assess for significant differences between the control and DLB groups. A summary of data, including medians and interquartile ranges for each group, is shown in table 4.9.

**Table 4.9: Summary of protein loads in grey matter for CIDL**

| Marker | Control median % [IQR] | DLB median % [IQR] | P value |
|---|---|---|---|
| **Aβ** | 0.543 [0.119-0.802] | 0.914 [0.263-1.496] | **0.039** * |
| **Ptau** | 0.023 [0.013-0.047] | 0.042 [0.019-0.068] | **0.031** * |
| **Iba1** | 0.931 [0.408-2.037] | 1.128 [0.803-1.694] | 0.537 |
| **HLA-DR** | 0.325 [0.249-1.669] | 0.436 [0.227-1.146] | 0.943 |
| **CD68** | 0.075 [0.051-0.139] | 0.116 [0.060-0.223] | 0.118 |
| **CD64** | 2.482 [1.722-2.880] | 2.580 [1.616-3.309] | 0.582 |
| **CD32a** | 0.491 [0.139-0.901] | 0.184 [0.086-0.578] | **0.043** * |
| **CD32b** | 0.076 [0.033-0.123] | 0.074 [0.031-0.211] | 0.705 |
| **CD16** | 0.091 [0.059-0.159] | 0.146 [0.098-0.283] | **0.027** * |
| **IL4R** | 0.411 [0.171-0.854] | 0.332 [0.174-0.616] | 0.912 |
| **CHI3L1** | 0.571 [0.238-0.823] | 0.344 [0.225-0.695] | 0.276 |

P value calculated using Mann-Whitney U test
*P<0.05 and in bold

### 4.2.4 Lewy-related pathology

Immunostaining for α-syn was analysed in the grey matter using a semi-quantitative assessment. Section 4.1.7 provides further information on this method of quantification, with figure 4.6 showing images representative of a score of 0 (no LRP) and 4 (very severe LRP).

Example digital images of tissue immunostained for α-syn are shown in figure 4.5 below, demonstrating LB and LN. There was significantly more LRP in the DLB group compared with the control group, as determined by Pearson Chi-squared test ($\chi^2(4)=48.122$, $P<0.001$). Data is presented graphically in figure 4.7.

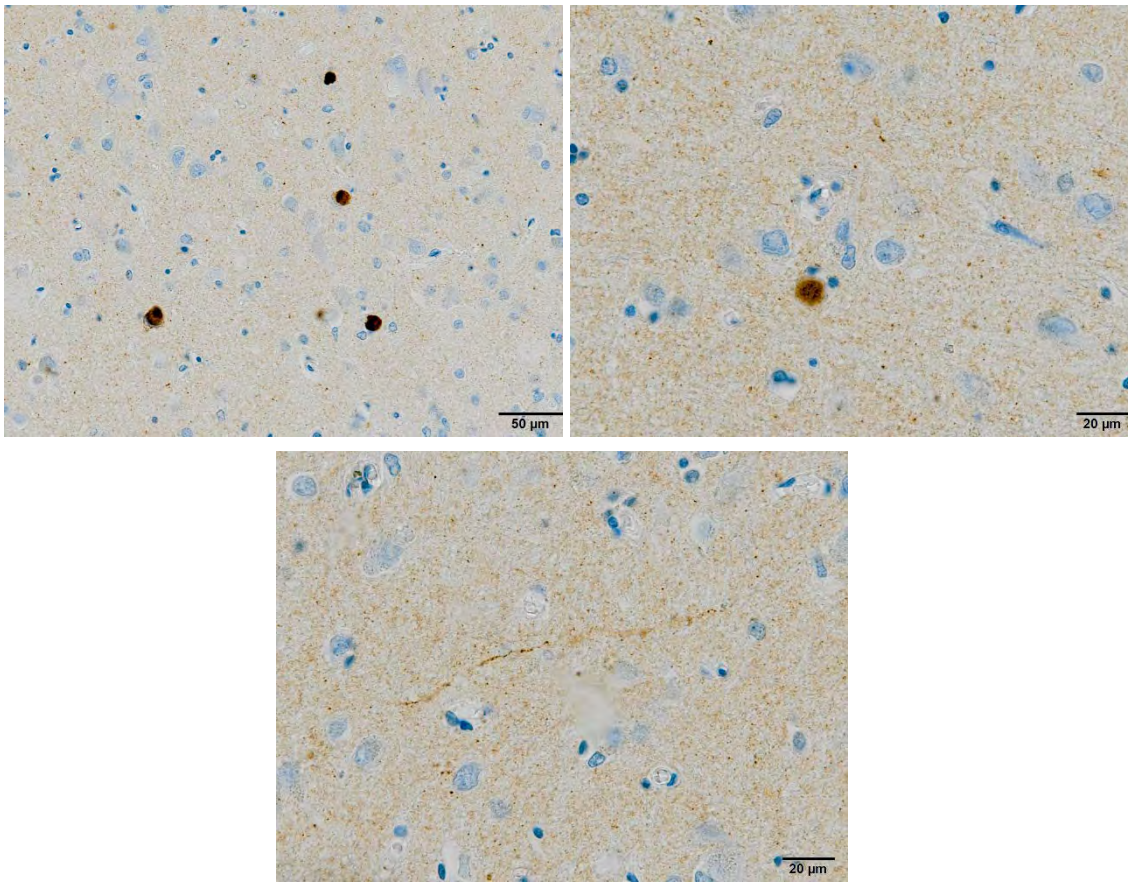



**Figure 4.5: α-syn immunostaining**
Example digital images taken of α-syn immunostaining. Multiple Lewy bodies (top left, x20). Single Lewy body at higher magnification (top right, x40). Lewy neurite (bottom, x40)

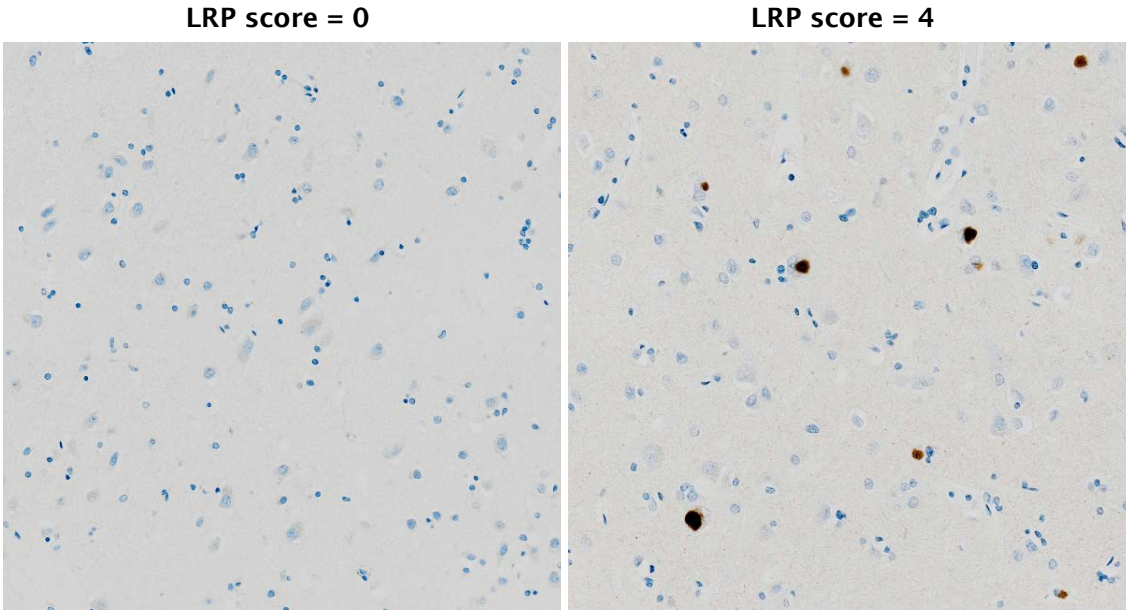



**Figure 4.6: Scoring of α-syn immunostaining as LRP in grey matter**
Example digital images taken of α-syn immunostaining (x20 magnification). No LRP (scored 0) on left and very severe LRP (scored 4) on right.

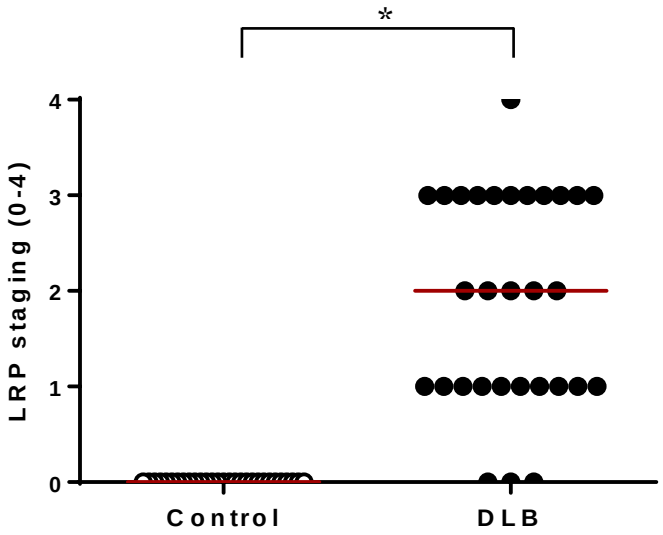



**Figure 4.7: LRP semi-quantitative analysis in grey matter**
Scatter plot showing LRP semi-quantitative staging in controls and DLB. All control groups had no LRP while there was a variable level of pathology in the DLB group. *P<0.001, Chi-squared test. Red line represents median.

### 4.2.5 AD pathology

Example images of AD pathology from DLB cases are shown in figures 4.8 and 4.10. Aβ protein load was higher in the grey matter of DLB cases compared to control cases (DLB median 0.914 [IQR 0.263 – 1.500] c.f. control median 0.534 [IQR 0.119 – 0.802], P=0.039, Mann Whitney U test), as shown in figure 4.9.

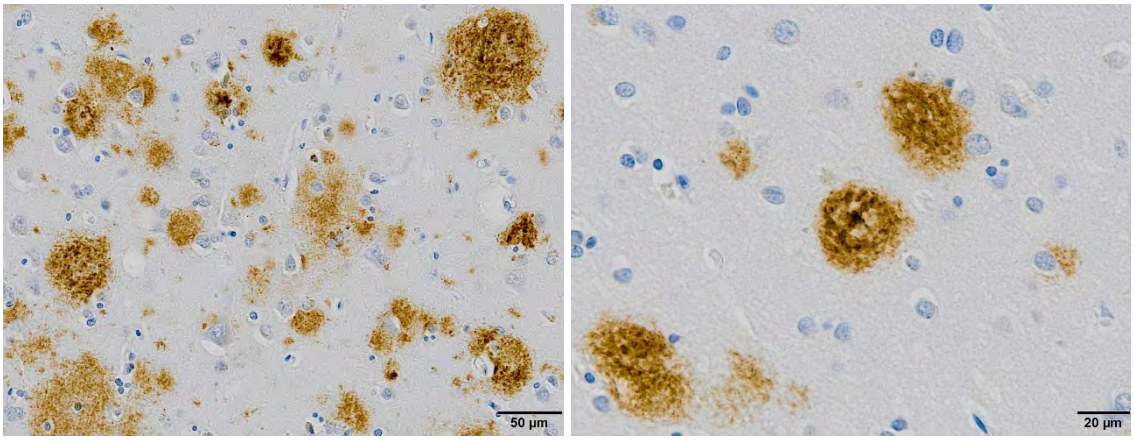



**Figure 4.8: Aβ immunostaining**
Example digital images taken of Aβ immunostaining. Diffuse amyloid plaques (left, x20). Dense core plaque (right, x40).

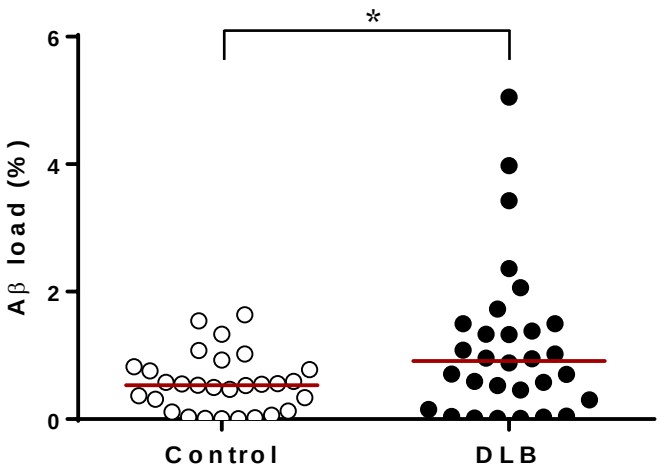



**Figure 4.9: Scatter plot of Aβ protein load in grey matter**
Scatter plot showing Aβprotein load in controls and DLB. Aβ protein load is significantly higher in DLB compared to controls. *P=0.039. Red line represents median.

The ptau protein load was higher in the grey matter of DLB cases compared to controls (DLB median 0.042 [IQR 0.019 – 0.068] c.f. control median 0.023 [IQR 0.013 – 0.047], P=0.031, Mann Whitney U test), as shown in figure 4.11.

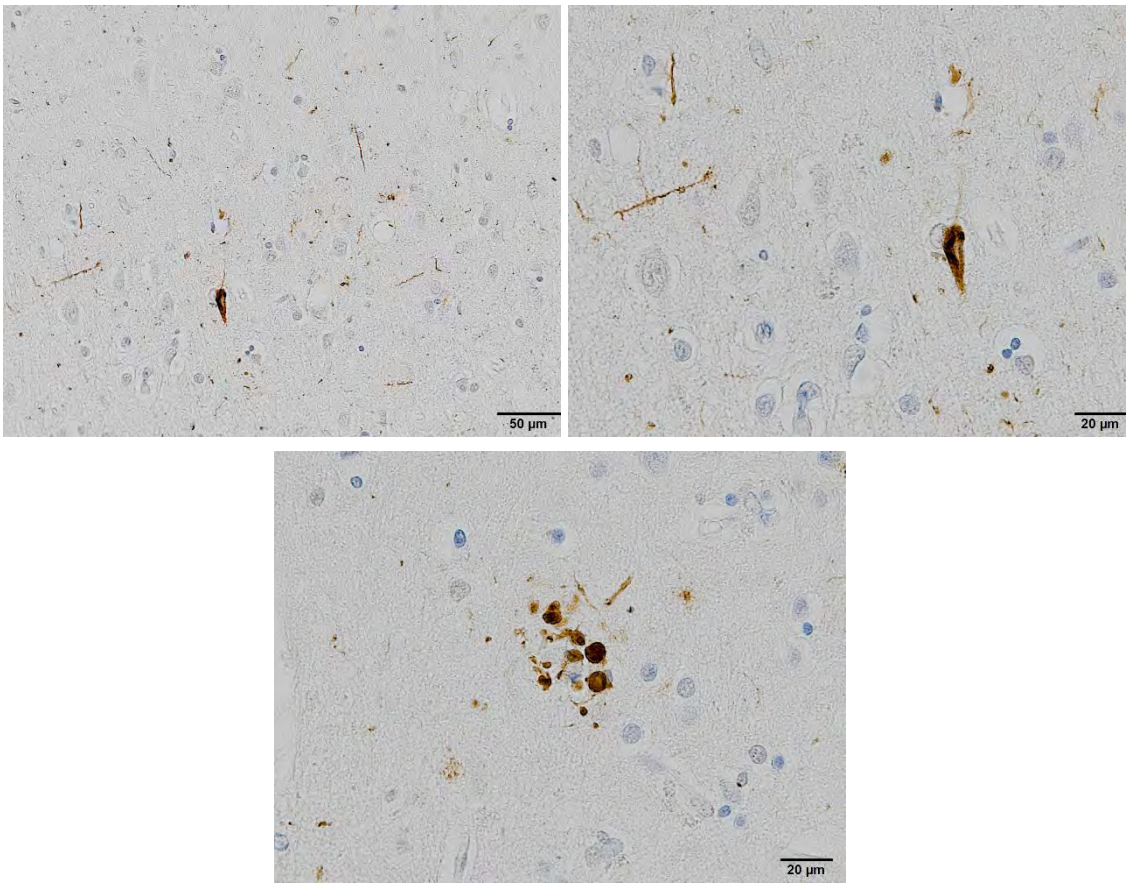



**Figure 4.10: Ptau immunostaining**
Example digital images of ptau immunostaining. Single ptau neurofibrillary tangle and multiple neuropil threads (top left at x20 and top right at x40). Cluster of ptau neuronal staining defined as dystrophic neurites (bottom, x40).

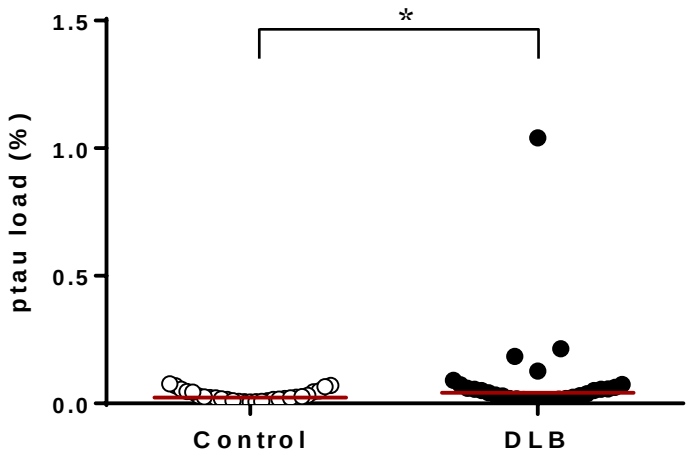



**Figure 4.11: Scatter plot of ptau protein load in grey matter**
Scatter plot showing ptau protein load in controls and DLB. Ptau protein load is significantly higher in DLB compared to controls. *P=0.031. Red line represents median.

### 4.2.6 Microglial markers in grey matter

Protein loads for the microglial markers Iba1, HLA-DR and CD68 in the grey matter were assessed for differences between the control and DLB groups. Example images of immunostained tissue from DLB cases are shown in figure 4.12. No group differences were found to be statistically significant, as presented below.

There was no significant difference in Iba1 protein load between DLB and control cases (DLB median 1.128 [IQR 0.803 – 1.694] c.f. control median 0.931 [IQR 0.408 – 2.037], P=0.537, Mann Whitney U test), as shown in figure 4.13.

There was no significant difference in HLA-DR protein load between DLB and control cases (DLB median 0.436 [IQR 0.227 – 1.146] c.f. control median 0.325 [IQR 0.249 – 1.146], P=0.943, Mann Whitney U test), as shown in figure 4.14.

Lastly, there was no significant difference in CD68 protein load between DLB and control cases (DLB median 0.116 [IQR 0.060 – 0.223] c.f. control median 0.075 [IQR 0.051 – 0.139], P=0.118, Mann Whitney U test), as shown in figure 4.15.

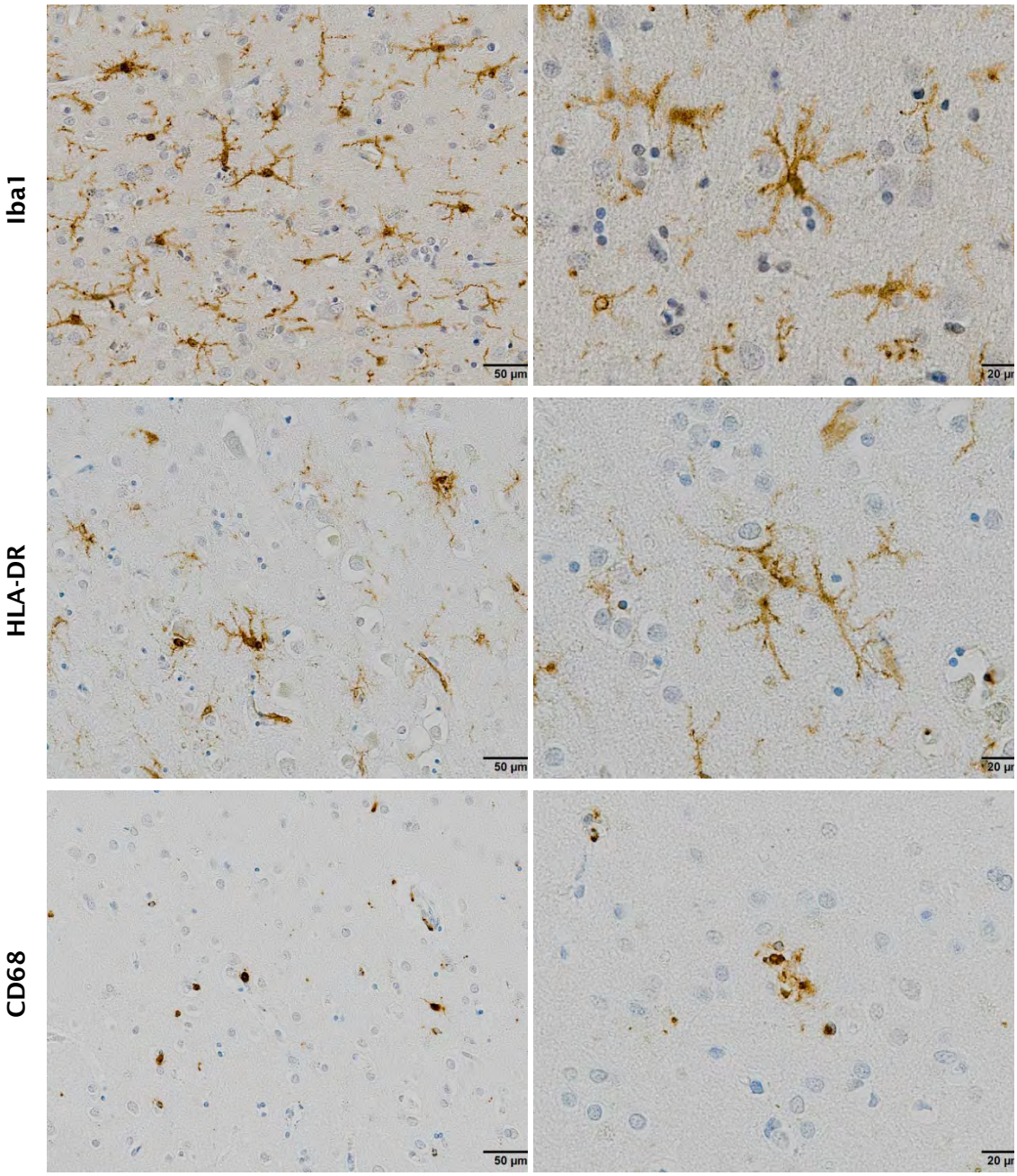



**Figure 4.12: Iba1, HLA-DR and CD68 immunostaining**
Example digital images of microglial immunostaining. Iba1 staining showing multiple ramified microglia (top left at x20 and top right at x40). HLA-DR staining showing multiple microglia (middle left x20) and clustering of microglia (middle right at x40). CD68 staining showing cytoplasmic staining (bottom left at x20) and clustering of cells (bottom right at x40).

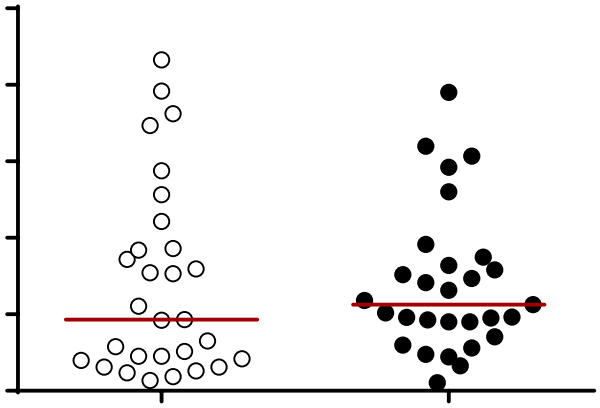


**Figure 4.13: Scatter plot of Iba1 protein load in grey matter**
Scatter plot showing Iba1 protein load in controls and DLB. No significant difference in protein load between groups. P=0.537. Red line represents median.

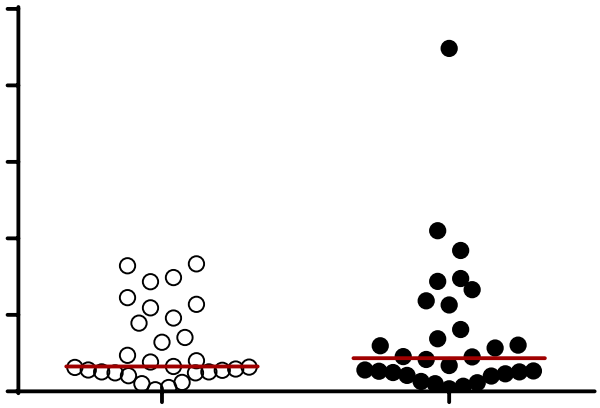


**Figure 4.14: Scatter plot of HLA-DR protein load in grey matter**
Scatter plot showing Iba1 protein load in controls and DLB. No significant difference in protein load between groups. P=0.943. Red line represents median.

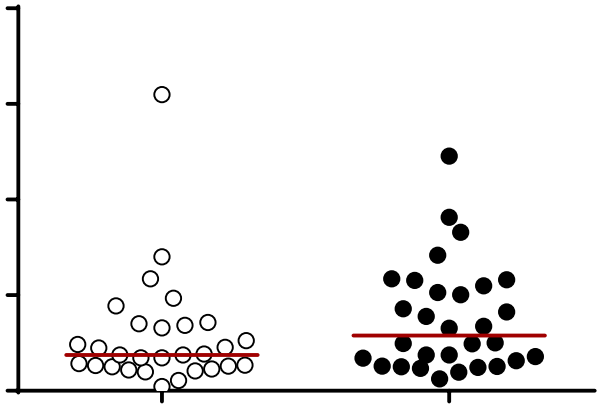


**Figure 4.15: Scatter plot of CD68 protein load in grey matter**
Scatter plot showing CD68 protein load in controls and DLB. No significant difference in protein load between groups. P=0.118. Red line represents median.

### 4.2.7 FcγR markers in grey matter

Immunostaining for FcγR revealed no inter-group differences in protein load for CD64 (P=0.582) or CD32b (P=0.705). CD32a protein load was significantly lower in DLB compared with controls (P=0.043), whilst CD16 protein load was significantly higher in DLB (P=0.027).

Figure 4.16 shows example images of DLB cases immunostained for FcγR. Data for the findings above are shown graphically in figures 4.17, 4.18, 4.19 and 4.20.

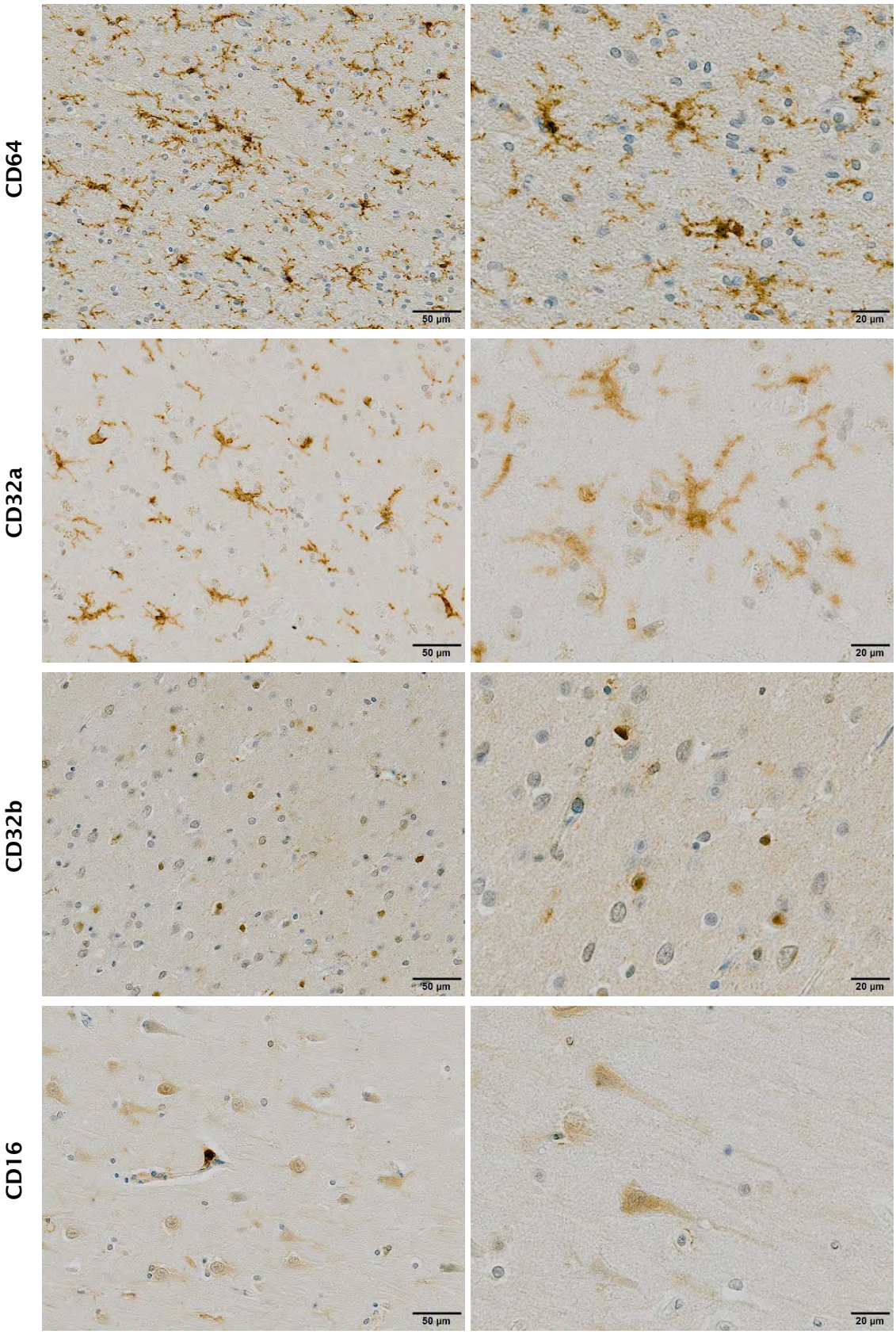



**Figure 4.16: FcγR immunostaining**
Example digital images of FcγR immunostaining. CD64 (top row, x20 of left and x40 on right). CD32a (second row, x20 on left and x40 on right). CD32b (third row, x20 on left and x40 on right). CD16 (bottom row, x20 on left and x40 on right).

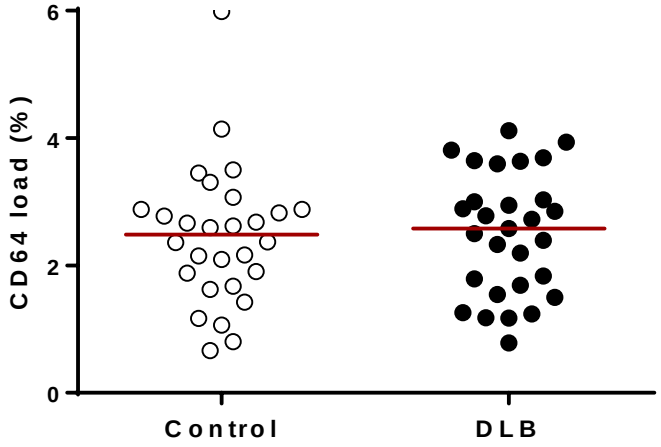



**Figure 4.17: Scatter plot of CD64 protein load in grey matter**
Scatter plot showing CD64 protein load in controls and DLB. No significant difference in protein load between groups. P=0.582. Red line represents median.

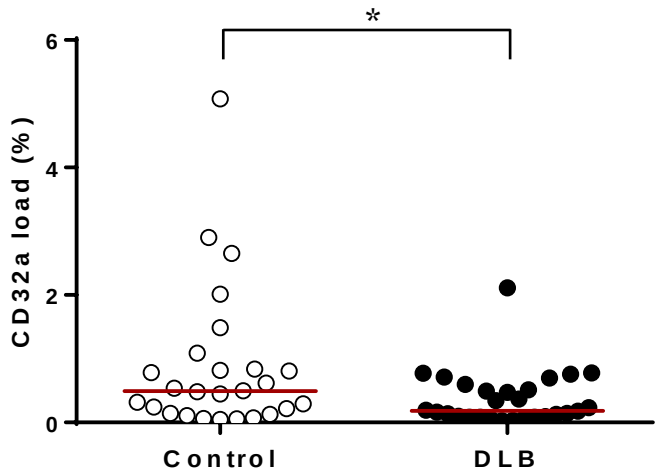



**Figure 4.18: Scatter plot of CD32a protein load in grey matter**
Scatter plot showing CD32a protein load in controls and DLB. CD32a protein load was significantly lower in DLB compared with controls. *P=0.043. Red line represents median.

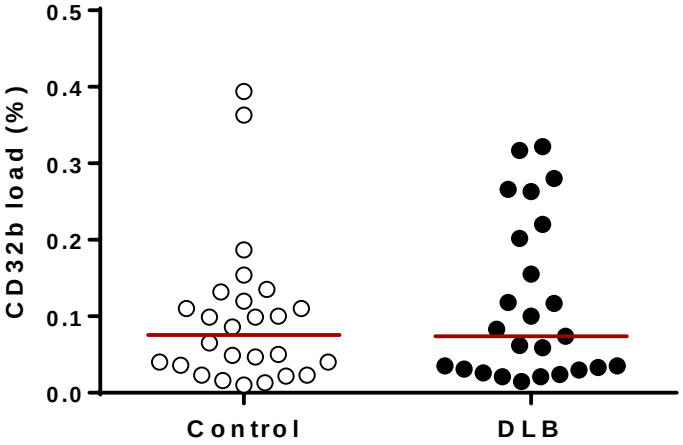



**Figure 4.19: Scatter plot of CD32b protein load in grey matter**
Scatter plot showing CD32b protein load in controls and DLB. No significant difference in protein load between groups. P=0.705. Red line represents median.

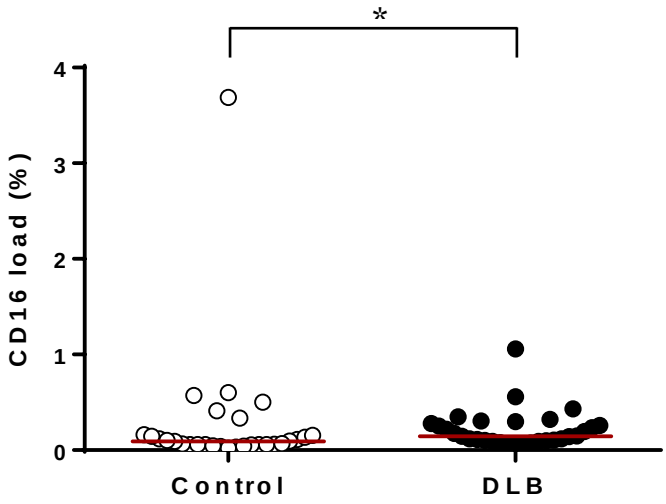



**Figure 4.20: Scatter plot of CD16 protein load in grey matter**
Scatter plot showing CD16 protein load in controls and DLB. CD16 protein load was significantly higher in DLB compared with controls. *P=0.027. Red line represents median.

### 4.2.8 Anti-inflammatory markers in grey matter

Analysis of immunostaining for the anti-inflammatory marker CHI3L1 revealed no significant difference in protein load between DLB and controls (P=0.276). IL4R protein load was also not significantly different between DLB and controls (P=0.912).

Example images of immunostained tissue in DLB cases are shown in figure 4.21. Data is presented graphically in figures 4.22 and 4.23.

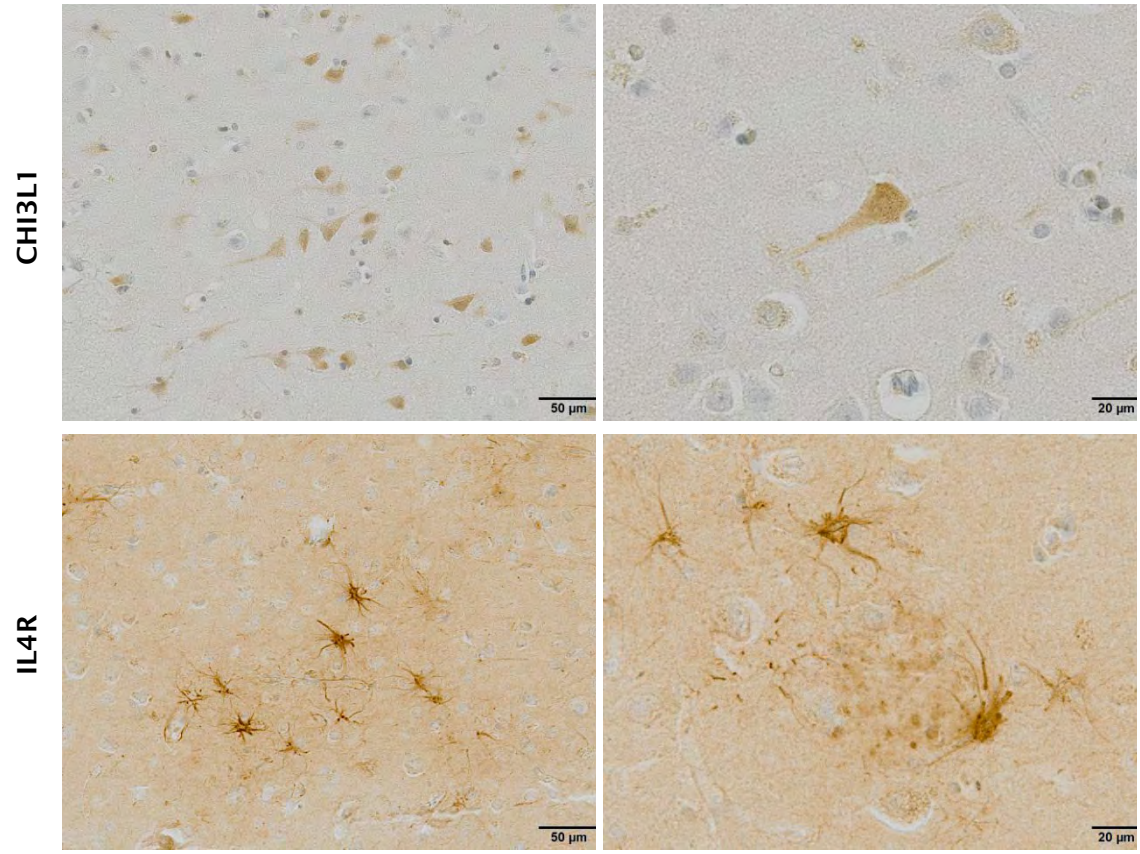



**Figure 4.21: Anti-inflammatory marker immunostaining**
Example digital images of anti-inflammatory marker immunostaining. CHI3L1 staining showing neuronal cytoplasmic staining (top left at x20 and top right at x40). IL4R staining showing multiple astrocytes (bottom left at x20) and clustering of astrocytes (bottom right at x40).

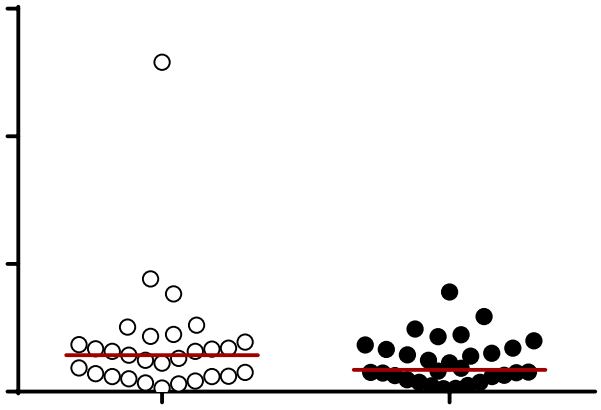


**Figure 4.22: Scatter plot of CHI3L1 protein load in grey matter**
Scatter plot showing CHI3L1 protein load in controls and DLB. No significant difference in protein load between groups. P=0.276. Red line represents median.

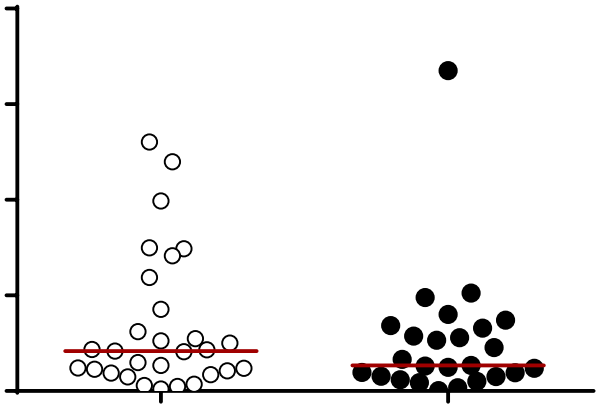


**Figure 4.23: Scatter plot of IL4R protein load in grey matter**
Scatter plot showing IL4R protein load in controls and DLB. No significant difference in protein load between groups. P=0.912. Red line represents median

### 4.2.9 Inflammatory markers in white matter

Digital images of the white matter were taken of brain tissue immunostained for inflammatory markers. White matter protein load was assessed for differences between the DLB and control groups. IL4R was not quantified in the white matter as immunohistochemistry revealed no specific staining.

No significant difference between groups was found in white matter protein load for any inflammatory marker. Data are summarised in table 4.10.

**Table 4.10: Summary of protein loads in white matter for CIDL**

| Marker | Control median % [IQR] | DLB median % [IQR] | P value |
|---|---|---|---|
| **Iba1** | 1.861 [0.843-2.529] | 1.650 [1.028-3.051] | 0.834 |
| **HLA-DR** | 1.289 [0.742-2.302] | 1.049 [0.527-1.914] | 0.291 |
| **CD68** | 0.311 [0.205-0.436] | 0.301 [0.206-0.583] | 0.534 |
| **CD64** | 4.223 [3.516-4.821] | 3.703 [3.034-5.404] | 0.652 |
| **CD32a** | 0.203 [0.080-0.715] | 0.111 [0.052-0.324] | 0.092 |
| **CD32b** | 0.005 [0.003-0.011] | 0.011 [0.005=0.025] | 0.082 |
| **CD16** | 0.338 [0.247-0.533] | 0.374 [0.229=0.675] | 0.525 |
| **CHI3L1** | 0.148 [0.062-0.660] | 0.126 [0.039-0.307] | 0.250 |

Significance is *P<0.05 and in bold

### 4.2.10 Correlation of inflammatory markers between grey and white matter

Protein loads in the grey and white matter for each inflammatory marker were assessed for correlation, separately for DLB and control groups. Spearman's rank correlations were used for all markers.

In the control group, there were positive and highly significant correlations for all inflammatory markers between the grey and white matter. All correlations between grey and white matter were also positive and highly significant in DLB, with the exception of HLA-DR. Data showing the Spearman's Rank correlation co-efficients and P values are presented in table 4.11 (controls) and 4.12 (DLB).

**Table 4.11: Control group – correlation between grey and white matter**

| Marker | Correlation co-efficient | P value |
|---|---|---|
| **Iba1** | 0.831 | **<0.001 *** |
| **HLA-DR** | 0.659 | **<0.001 *** |
| **CD68** | 0.716 | **<0.001 *** |
| **CD64** | 0.612 | **0.001 *** |
| **CD32a** | 0.898 | **<0.001 *** |
| **CD32b** | 0.840 | **<0.001 *** |
| **CD16** | 0.768 | **<0.001 *** |
| **CHI3L1** | 0.763 | **<0.001 *** |

Significance is *P<0.05 and in bold

**Table 4.12: DLB group – correlation between grey and white matter**

| Marker | Correlation co-efficient | P value |
|---|---|---|
| **Iba1** | 0.824 | **<0.001 *** |
| **HLA-DR** | 0.124 | 0.529 |
| **CD68** | 0.726 | **<0.001 *** |
| **CD64** | 0.749 | **<0.001 *** |
| **CD32a** | 0.867 | **<0.001 *** |
| **CD32b** | 0.770 | **<0.001 *** |
| **CD16** | 0.673 | **<0.001 *** |
| **CHI3L1** | 0.765 | **<0.001 *** |

Significance is *P<0.05 and in bold

### 4.2.11 Correlation between neuropathological and inflammatory markers

Protein loads in the grey matter for all markers of neuropathology and inflammation were assessed for correlation. Spearman's rank correlations were used, with $P<0.01$ deemed significant to account for multiple correlations. In both groups, no significant correlations were found between any single marker of inflammation and any of the three markers of neuropathology.

In the control group, significant positive correlations were found for the following associations between inflammatory markers:

- CD68 and HLA-DR
- CD68 and CD64
- CD68 and CD16
- CD32a and Iba1
- CD32a and CD32b
- CD16 and CD64

In the DLB group, significant positive correlations were found for the following associations between inflammatory markers:

- CD68 and CD64 (as in the control group)
- CD68 and CD16 (as in the control group)
- CD32a and Iba1 (as in the control group)
- CD16 and CD64 (as in the control group)
- CD32b and CD64
- CD32b and CD68
- CD68 and IL4R
- HLA-DR and IL4R

Positive significant correlations between HLA-DR and CD68, and between CD32a and CD32b were present in the control group but lost in the DLB group. Correlation tables for both the control and DLB groups are shown in tables 4.13 and 4.14 respectively.

**Table 4.13: Control group: table of correlations of protein load between neuropathological and inflammatory markers**

| | | Aβ | Ptau | Iba1 | HLA-DR | CD68 | CD64 | CD32a | CD32b | CD16 | CHI3L1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aβ** | Coefficient | | | | | | | | | | |
| | Sig. (2-tailed) | | | | | | | | | | |
| **Ptau** | Coefficient | 0.136 | | | | | | | | | |
| | Sig. (2-tailed) | 0.482 | | | | | | | | | |
| **Iba1** | Coefficient | 0.294 | 0.147 | | | | | | | | |
| | Sig. (2-tailed) | 0.121 | 0.446 | | | | | | | | |
| **HLA-DR** | Coefficient | -.380* | -0.308 | -0.104 | | | | | | | |
| | Sig. (2-tailed) | 0.042 | 0.104 | 0.591 | | | | | | | |
| **CD68** | Coefficient | 0.083 | -0.171 | 0.047 | **.505** | | | | | | |
| | Sig. (2-tailed) | 0.670 | 0.375 | 0.809 | **0.005**** | | | | | | |
| **CD64** | Coefficient | -0.210 | -.420* | -0.265 | 0.346 | **.616** | | | | | |
| | Sig. (2-tailed) | 0.284 | 0.026 | 0.173 | 0.071 | **0.000**** | | | | | |
| **CD32a** | Coefficient | 0.000 | -0.030 | **.649** | 0.229 | 0.144 | -0.075 | | | | |
| | Sig. (2-tailed) | 0.999 | 0.885 | **0.000**** | 0.261 | 0.484 | 0.722 | | | | |
| **CD32b** | Coefficient | 0.141 | 0.290 | 0.114 | -0.118 | 0.051 | -0.108 | **.604** | | | |
| | Sig. (2-tailed) | 0.511 | 0.170 | 0.597 | 0.582 | 0.813 | 0.623 | **0.003**** | | | |
| **CD16** | Coefficient | -0.045 | -0.253 | -0.095 | 0.171 | **.522** | **.570** | 0.039 | 0.243 | | |
| | Sig. (2-tailed) | 0.817 | 0.186 | 0.625 | 0.375 | **0.004**** | **0.002**** | 0.850 | 0.253 | | |
| **CHI3L1** | Coefficient | 0.341 | 0.343 | .400* | -0.099 | 0.243 | -0.071 | 0.348 | 0.282 | 0.216 | |
| | Sig. (2-tailed) | 0.070 | 0.068 | 0.032 | 0.609 | 0.204 | 0.718 | 0.081 | 0.183 | 0.260 | |
| **IL4R** | Coefficient | -0.188 | 0.121 | 0.210 | 0.035 | 0.003 | -0.109 | 0.376 | 0.119 | -0.107 | 0.153 |
| | Sig. (2-tailed) | 0.330 | 0.532 | 0.275 | 0.858 | 0.988 | 0.582 | 0.059 | 0.580 | 0.579 | 0.427 |

Results are Spearman's rank correlation co-efficient
*P<0.05, **P<0.01 and in bold/shaded

**Table 4.14: DLB group: table of correlations of protein load between neuropathological and inflammatory markers**

| | | α-syn | Aβ | Ptau | Iba1 | HLA-DR | CD68 | CD64 | CD32a | CD32b | CD16 | CHI3L1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **α-syn** | Coefficient | | | | | | | | | | | |
| | Sig. (2-tailed) | | | | | | | | | | | |
| **Aβ** | Coefficient | .410* | | | | | | | | | | |
| | Sig. (2-tailed) | 0.024 | | | | | | | | | | |
| **Ptau** | Coefficient | 0.284 | 0.352 | | | | | | | | | |
| | Sig. (2-tailed) | 0.135 | 0.061 | | | | | | | | | |
| **Iba1** | Coefficient | -0.058 | -0.198 | -0.050 | | | | | | | | |
| | Sig. (2-tailed) | 0.764 | 0.303 | 0.800 | | | | | | | | |
| **HLA-DR** | Coefficient | 0.262 | -0.050 | 0.257 | -0.219 | | | | | | | |
| | Sig. (2-tailed) | 0.162 | 0.795 | 0.178 | 0.253 | | | | | | | |
| **CD68** | Coefficient | 0.205 | 0.091 | .376* | -0.201 | .399* | | | | | | |
| | Sig. (2-tailed) | 0.278 | 0.634 | 0.045 | 0.296 | 0.029 | | | | | | |
| **CD64** | Coefficient | 0.018 | 0.168 | 0.219 | -0.122 | 0.226 | **.550** | | | | | |
| | Sig. (2-tailed) | 0.924 | 0.382 | 0.263 | 0.538 | 0.238 | **0.002**** | | | | | |
| **CD32a** | Coefficient | 0.104 | -0.259 | 0.128 | **.604** | 0.128 | 0.103 | 0.265 | | | | |
| | Sig. (2-tailed) | 0.599 | 0.182 | 0.525 | **0.001**** | 0.516 | 0.603 | 0.182 | | | | |
| **CD32b** | Coefficient | -0.043 | -0.149 | -0.052 | -0.011 | 0.084 | **.540** | **.501** | 0.346 | | | |
| | Sig. (2-tailed) | 0.833 | 0.457 | 0.799 | 0.957 | 0.676 | **0.004**** | **0.009**** | 0.083 | | | |
| **CD16** | Coefficient | 0.264 | 0.082 | 0.195 | -0.277 | 0.360 | **.481** | **.648** | 0.051 | 0.325 | | |
| | Sig. (2-tailed) | 0.158 | 0.668 | 0.311 | 0.146 | 0.051 | **0.007**** | **0.000**** | 0.796 | 0.098 | | |
| **CHI3L1** | Coefficient | -0.081 | .395* | 0.235 | -0.208 | -0.275 | 0.008 | 0.188 | -0.227 | -0.098 | -0.012 | |
| | Sig. (2-tailed) | 0.669 | 0.031 | 0.220 | 0.279 | 0.142 | 0.967 | 0.330 | 0.246 | 0.625 | 0.949 | |
| **IL4R** | Coefficient | 0.125 | 0.028 | 0.381 | -0.028 | **.682** | **.617** | .514* | 0.165 | .456* | 0.308 | -0.117 |
| | Sig. (2-tailed) | 0.552 | 0.895 | 0.060 | 0.897 | **0.000**** | **0.001**** | 0.010 | 0.453 | 0.029 | 0.135 | 0.578 |

Results are Spearman's rank correlation co-efficient
*P<0.05, **P<0.01 and in bold/shaded

The data shown in the preceding correlation tables are summarised below, in table 4.15. This illustrates correlations between inflammatory markers, enabling a straightforward comparison between the control group (top) and DLB group (bottom). Positive correlations are shaded in green. No negative correlations were found.

**Table 4.15: Illustration of correlations between inflammatory markers**

| **CONTROL** | Iba1 | HLA-DR | CD68 | CD64 | CD32a | CD32b | CD16 | CHI3L1 | IL4R |
|---|---|---|---|---|---|---|---|---|---|
| Iba1 | - | - | - | - | - | - | - | - | - |
| HLA-DR | | - | - | - | - | - | - | - | - |
| CD68 | | | - | - | - | - | - | - | - |
| CD64 | | | | - | - | - | - | - | - |
| CD32a | | | | | - | - | - | - | - |
| CD32b | | | | | | - | - | - | - |
| CD16 | | | | | | | - | - | - |
| CHI3L1 | | | | | | | | - | - |
| IL4R | | | | | | | | | - |

| **DLB** | Iba1 | HLA-DR | CD68 | CD64 | CD32a | CD32b | CD16 | CHI3L1 | IL4R |
|---|---|---|---|---|---|---|---|---|---|
| Iba1 | - | - | - | - | - | - | - | - | - |
| HLA-DR | | - | - | - | - | - | - | - | - |
| CD68 | | | - | - | - | - | - | - | - |
| CD64 | | | | - | - | - | - | - | - |
| CD32a | | | | | - | - | - | - | - |
| CD32b | | | | | | - | - | - | - |
| CD16 | | | | | | | - | - | - |
| CHI3L1 | | | | | | | | - | - |
| IL4R | | | | | | | | | - |

### 4.2.12 Comparison with AD group

As described in the methods, an AD group was not included in CIDL as the microglial phenotype in AD had already been examined by the Boche group in Southampton (publication in preparation). This work was completed by Dr Sonja Rakic, who provided permission for a brief summary of this work to be presented below.

Post-mortem human brain tissue from the inferior parietal lobule was obtained from 68 AD cases and 40 controls, provided by South West Dementia Brain Bank (University of Bristol) and BRAIN UK (Queen Elizabeth University Hospital Glasgow). Immunohistochemistry and analysis was performed using the same methods as presented in section 4.1. Importantly, antibodies and experimental conditions were the same between CIDL and the AD study.

A summary of data from this study is presented in table 4.16. All variables were found to be non-normal in distribution according to Shapiro-Wilk test results. Therefore, the Mann-Whitney U test was used to assess for significant differences in protein load between controls and AD. Two-tailed P values of less than 0.05 were deemed to be significant.

Overall, the study showed increased neuropathology in the AD group compared with controls, with significantly higher protein loads for both Aβ and ptau in the AD group. With regards to microglial markers, CD68 protein load was significantly higher in AD, but Iba1 and HLA-DR protein loads were not different. The activating FcγR CD64 and CD16 were increased in AD, whilst the inhibitory CD32b was reduced in AD. Both anti-inflammatory markers, IL4R and CHI3L1, were significantly increased in AD compared with controls.

**Table 4.16: Summary of protein loads of all markers in AD study**

| Marker | Control median % [IQR] | AD median % [IQR] | P value |
|---|---|---|---|
| **Aβ** | 1.036 [0.118-4.572] | 6.367 [4.819-8.625] | **<0.001** * |
| **Ptau** | 0.004 [0.002-0.012] | 1.201 [0.487-2.765] | **<0.001** * |
| **Iba1** | 1.554 [0.997-2.207] | 1.204 [0.526-1.965] | 0.058 |
| **HLA-DR** | 0.013 [0.003-0.036] | 0.023 [0.004-0.135] | 0.135 |
| **CD68** | 0.228 [0.160-0.277] | 0.251 [0.207-0.330] | **0.033** * |
| **CD64** | 2.165 [1.325-2.874] | 2.728 [1.896-3.941] | **0.002** * |
| **CD32a** | 0.324 [0.147-0.599] | 0.241 [0.029-0.553] | 0.131 |
| **CD32b** | 0.075 [0.008-0.130] | 0.059 [0.015-0.232] | **0.044** * |
| **CD16** | 0.224 [0.088-0.799] | 0.411 [0.195-1.182] | **0.014** * |
| **IL4R** | 0.052 [0.026-0.112] | 0.112 [0.045-0.244] | **0.001** * |
| **CHI3L1** | 0.151 [0.073-0.441] | 0.340 [0.173-0.859] | **<0.001** * |

Significance is *P<0.05 and in bold
Data from Dr Sonja Rakic (unpublished)

Data were further assessed for statistically significant associations between all markers using the Spearman's rank test, with findings deemed significant if P<0.01 to allow for multiple comparisons. Table 4.17 illustrates correlations between inflammatory markers, allowing for comparison between the control group (top) and AD group (bottom). Positive correlations are shaded in green. No negative correlations were found.

**Table 4.17: Illustration of correlations between inflammatory markers in AD study**

| CONTROL | Aβ | ptau | Iba1 | HLA-DR | CD68 | CD64 | CD32a | CD32b | CD16 | CHI3L1 | IL4R |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Aβ | - | - | - | - | - | - | - | - | - | - | - |
| ptau | | - | - | - | - | - | - | - | - | - | - |
| Iba1 | | | - | - | - | - | - | - | - | - | - |
| HLA-DR | | | | - | - | - | - | - | - | - | - |
| CD68 | | | | | - | - | - | - | - | - | - |
| CD64 | | | | | | - | - | - | - | - | - |
| CD32a | | | | | | | - | - | - | - | - |
| CD32b | | | | | | | | - | - | - | - |
| CD16 | | | | | | | | | - | - | - |
| CHI3L1 | | | | | | | | | | - | - |
| IL4R | | | | | | | | | | | - |

| AD | Aβ | ptau | Iba1 | HLA-DR | CD68 | CD64 | CD32a | CD32b | CD16 | CHI3L1 | IL4R |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Aβ | - | - | - | - | - | - | - | - | - | - | - |
| ptau | | - | - | - | - | - | - | - | - | - | - |
| Iba1 | | | - | - | - | - | - | - | - | - | - |
| HLA-DR | | | | - | - | - | - | - | - | - | - |
| CD68 | | | | | - | - | - | - | - | - | - |
| CD64 | | | | | | - | - | - | - | - | - |
| CD32a | | | | | | | - | - | - | - | - |
| CD32b | | | | | | | | - | - | - | - |
| CD16 | | | | | | | | | - | - | - |
| CHI3L1 | | | | | | | | | | - | - |
| IL4R | | | | | | | | | | | - |

## 4.3 Conclusions

The aim of this study was to determine the microglial immunophenotype in DLB compared to controls. Immunohistochemistry was used to investigate a number of markers of pathology and inflammation in post-mortem human brain tissue. Furthermore, markers of inflammation were tested for associations with the neuropathological features of DLB. Lastly, data were compared with a related study that examined microglial phenotype in AD.

### 4.3.1 Baseline characteristics

Baseline analysis of cases included in CIDL revealed that the DLB and control groups were well matched for age, gender, post-mortem delay and Braak ptau stage. There was no difference in *APOE* genotype between groups, however this was potentially not representative of true population differences as limited genotyping data was received from brain banks, resulting in poor statistical power.

The mean duration of disease for DLB cases was 6.7 years, showing that the majority of participants were substantially into their disease course at the time of death.

### 4.3.2 Neuropathology in DLB

Analysis of neuropathology data in CIDL showed increased LRP and AD pathology in the DLB brain compared with controls, entirely as expected and in keeping with previous literature [67-69]. The presence of LRP in the DLB group, and its absence in all control cases, confirmed the accuracy of case selection. Interestingly it has been shown previously that there is a lack of association between cerebral Aβ load and clinical diagnosis of DLB or AD, implying that the presence of cortical Aβ alone cannot differentiate the two diseases [396]. Despite the expected overlap of AD pathology into the DLB group, it should be noted that DLB cases with significant ptau pathology were excluded during case selection to prevent inclusion of mixed AD and DLB cases. This was done by rejecting DLB cases with Braak ptau scores greater than 3. As explored in the introduction, the areas of overlap between AD and DLB includes not only neuropathology but also genetics, neurochemistry and clinical features.

### 4.3.3 Microglial immunophenotype in DLB

Many previous studies in DLB utilised just one or two microglial markers, preventing them from examining the phenotype of microglia. Iba1, HLA-DR and CD68 have been used extensively to investigate activation of microglia in AD [169]. Iba1 is a microglial marker associated with cell motility, HLA-DR is a marker of antigen presentation and CD68 is a marker of phagocytosis.

The primary finding of CIDL is that when compared with controls, there was no difference in the levels of the three classical markers of microglial activation in DLB. This finding supports some of the previous literature in this area, which has found no alteration in the expression of HLA-DR [327] or Iba1 in DLB [330, 331]. The Streit et al. study [330] is particularly comparable to CIDL as the same Iba1 antibody and method of quantification was used, and the same brain area was studied. Whilst it showed no change in Iba1 protein load, it did find higher CD68 protein load in DLB compared to controls, whereas our results showed no significant difference. Streit et al. appeared to downplay this finding, attributing the increase in CD68 to lipofuscin deposits, especially in the absence of detection of any alteration in microglial morphology in DLB compared with controls [330].

Two previous studies have found results that are contrary to the findings from CIDL, both demonstrating increased HLA-DR-positive microglia in DLB [326, 329]. The contrast between findings from CIDL and the Mackenzie study [326] is particularly challenging to assess due to the lack of detail that the published article contains regarding case selection and analysis. Mackenzie reports that DLB cases were excluded if they had significant “senile plaque” pathology but it is unclear as to whether they had significant ptau pathology, which if present could have contributed to increased HLA-DR-positive microglia in DLB. The Imamura et al. study [329] appeared to examine a very small area of the brain (five x200 magnification fields of five different sections of the hippocampus, amygdala and transentorhinal cortex) and in a very limited number of cases (5 DLB and 4 controls). It may therefore have been susceptible to sampling bias.

The most recent study published in this area is by Erskine et al. [332]. It showed no change in the level of protein expression of Iba1 or HLA-DR in DLB upon microscopy of immunostained tissue in the pulvinar, using broadly

similar quantification techniques to SILAD. Interestingly, it did show increased mRNA levels and protein load of glial fibrillary acidic protein (GFAP), an astrocytic marker, and decreased expression of multiple pre-synaptic proteins in DLB. The authors concluded that there was no evidence of increased activity of microglia in this area of the brain, but that astrogliosis may play a role in supporting deteriorating synaptic function in DLB. The lack of increase in Iba1 and HLA-DR in DLB found in CIDL appears to be consistent with the results of the Erskine et al. study.

It is noteworthy that the cohort of 30 DLB cases and 29 control cases in CIDL far exceeds the number of cases previously examined in DLB, and therefore should be considered to be more powered to detect significant differences than those studies above. Taking all of these studies into consideration, including their relative strengths and weaknesses, the findings from CIDL pertaining to the three classical microglial markers supports the majority of previous literature in DLB.

One possible reason why the classical markers of microglial activation (Iba1, CD68 and HLA-DR) were not increased in DLB could relate to the limited overall burden of neuropathology in DLB. Despite being the pathological hallmark of the disease, LRP was noted to be relatively sparse even in the most severe DLB cases. It has been proposed that the primary pathogenic feature in DLB is not LRP but instead pre-synaptic α-syn aggregates, which may cause neurochemical imbalance and synaptic dysfunction, meaning that they could be the real perpetrators of symptomatology in DLB [72]. In addition, the extent of Aβ and ptau pathology present in DLB cases in CIDL was, whilst greater than that found in the control group, dramatically lower than that found in the AD study (Dr Sonja Rakic, unpublished). It may be that very high levels of AD pathology are required to drive neuroinflammation in DLB. This theory is supported by previous evidence showing increased temporal lobe neuronal loss in DLB cases possessing prominent AD pathology [70], and other work that has described worsening cognitive decline in DLB cases that possess severe concomitant AD pathology [67]. Furthermore, the severity of AD pathology found in DLB has been shown to be detrimental to prognosis in a robust and prospectively recruited post-mortem study [71]. Lastly, there is little evidence of extensive loss of cortical synapses in DLB without the presence of concurrent AD

pathology [397]. Overall, it can be hypothesised that the level of AD pathology is a key factor in the progression of neurodegeneration and dementia in DLB. LRP has not been shown to be associated with cognitive decline in DLB and may not induce significant neuroinflammation alone. Therefore, the synergistic effect of the interaction of AD pathology with synaptic α-syn aggregation may better explain the disease process in DLB and may be key in producing a neuroinflammatory response.

### 4.3.4 FcγR profile in DLB

The expression of FcγR has not been previously explored in DLB. These receptors play a key role in the activation of microglia and co-ordination of a phagocytic response, and have been shown to be expressed on microglial cells including those that cluster around amyloid plaques in AD [230]. In addition, increased CD64 expression has previously been identified in PD [335].

The FcγR CD64, CD32a and CD16 are known to be activating, producing a pro-inflammatory response, while CD32b is an inhibitory receptor. CD64 is known to have high affinity for IgG (including monomeric IgG), whereas CD32a and CD16 are low affinity receptors. The overall balance of activating versus inhibitory FcγR on the microglial cell surface dictates the activation level of the cell, and thus how strongly it will respond in the presence of IgG or immune complexes.

In this study, the profile of FcγR in DLB differed markedly to what has previously been reported in AD. No alteration in CD64 or CD32b was found in CIDL, whereas CD32a was lower and CD16 higher in DLB. The CD16 antibody used in the immunohistochemistry experiments actually targeted both CD16a and CD16b receptors. CD16a has an activating profile whereas CD16b is known to be a decoy receptor [173]. It is therefore not possible to confidently determine whether the increased protein load of CD16 found in DLB indicates a change in the activating and/or decoy profile of CD16. In addition, CD16 immunostaining was noted to localise primarily to small circular cells within blood vessel lumen, indicating immunoreactivity for monocytes or natural killer cells rather than microglia. Therefore, there may be a role in DLB for peripheral

monocytes or natural killer cells, possibly communicating with microglia via perivascular macrophages and endothelial cells in the BBB.

### 4.3.5 Anti-inflammatory markers in DLB

In addition to the markers discussed above, two anti-inflammatory markers were also examined. There was no difference detected in the expression of either CHI3L1 or IL4R in DLB compared with controls. Neither of these two markers have been previously examined in the DLB brain. CHI3L1 has previously been found to be expressed at increased levels in AD, particularly localising to astrocytes around amyloid plaques [263], and has also been shown to downregulate cellular responses to the pro-inflammatory cytokines IL1$\beta$ and TNF$\alpha$ *in-vitro* [398]. Meanwhile IL4 is a known anti-inflammatory cytokine that has been shown to trigger alternative activation of microglia [395]. In the periphery IL4 is also produced by cells of the adaptive immune system, leading to the development and maintenance of wound healing macrophages [164].

Both CHI3L1 and IL4 are markers associated with an immunosuppressive environment. Since there appears to be a lack of microglial activation in DLB, it follows that there may be no requirement for a reactionary anti-inflammatory response to keep neuroinflammation in check. This finding contrasts with results in AD, as discussed further in section 4.3.10.

### 4.3.6 Comparison with neuroimaging literature

The findings from CIDL do appear to contrast with published work on *in-vivo* imaging of inflammation, which has shown increased microglial activation in DLB compared to controls [314, 315]. One possible explanation for this is that the expression of TSPO, the target of the PK11195 ligand used in many imaging studies, may differ from the profile of the inflammatory activation markers assessed in CIDL. A limitation of using this ligand, which has already been discussed, is that it remains unclear whether the increased signal found in DLB is related to microglial or astrocytic activation [219, 220]. Furthermore, both imaging studies in DLB to date showed increased TSPO binding in the early stages of the disease, suggesting a role for cerebral inflammation in mild DLB that possibly reduces with time. The precise phenotype of glia in early DLB

may indeed prove to be significantly different to that found in CIDL, with a possible early role for astrogliosis in supporting synaptic function as pre-synaptic aggregates of α-syn start to disturb neurotransmission and trigger the onset of clinical symptoms.

### 4.3.7 Correlations between grey and white matter

Correlations between the grey and white matter for each inflammatory marker give an indication of the uniformity of microglial phenotype throughout the brain. All inflammatory markers showed significant positive correlations between grey and white matter in the control group, supporting the theory that such uniformity is beneficial. These findings remained in the DLB group apart from one marker, HLA-DR, which lost its significant correlation between grey and white matter. The primary function of HLA-DR is to present antigen to the adaptive immune system in order to produce a T cell response. The absence of an association of HLA-DR expression between grey and white matter in DLB suggests the lack of a common target between the two areas and a possible role for HLA-DR in DLB. It was noted that HLA-DR expression was higher in the white matter compared with the grey matter, suggesting a relative downregulation of the antigen presenting phenotype of microglia in DLB grey matter. This could reflect the low antigenic load in DLB grey matter, perhaps as a result of limited extracellular protein deposition in the disease. Alternatively, the strong HLA-DR signal noted in the white matter may represent increased detection of perivascular macrophages and not microglia, possibly explaining the lack of positive correlation of HLA-DR signal between grey and white matter in DLB.

### 4.3.8 Correlations between inflammatory markers

Correlations between inflammatory markers implies a level of co-ordination of microglia activation in response to stimuli. Review of the correlations between inflammatory markers in the control group revealed positive associations between markers of both antigen presentation (HLA-DR) and motility (Iba1) with a marker of phagocytosis (CD68). There were also numerous positive correlations between markers of phagocytic phenotype (CD68 and the activating FcγR). In addition, the positive correlation between CD32a and

CD32b supports the presence of a homeostatic equilibrium between activating and inhibitory FcγR in the healthy brain [173]. Overall, this high correlation of microglial markers in controls, potentially indicating different microglial populations, indicates co-ordination of inflammation and homeostasis in the healthy brain, which does not generate a damaging inflammatory reaction.

In DLB, the pattern of associations between inflammatory markers was altered. The correlations between HLA-DR and CD68, and between CD32a and CD32b, were both lost. All other correlations from the control group remained present in DLB. In addition, the inhibitory FcγR CD32b correlated positively with CD68 and CD64, both markers of a phagocytic phenotype. Interestingly, the anti-inflammatory marker IL4R was also found to correlate positively with both CD68 and HLA-DR. The change in pattern of correlations in DLB suggests that there may be a disturbance to the interactions in homeostatic inflammatory processes in DLB. The healthy brain appears to show co-ordination of different immune functions simultaneously, both activating and inhibitory. This equilibrium is likely to be essential in maintaining a neuroprotective environment. In contrast, the DLB brain shows relationships between phagocytic markers along with anti-inflammatory markers. This supports the theory that in DLB, the microglial sub-population showing a more prominent phagocytic phenotype is associated with an increased anti-inflammatory response, both via the inhibitory CD32b but also via IL4R. It may be that this response is an attempt to prevent excessive phagocytosis and is neuroprotective in DLB. Alternatively, it could be deleterious as microglia may not be able to phagocytose where needed due to the presence of an immunosuppressive, anti-inflammatory environment late in the disease process.

### 4.3.9 Correlations with neuropathology

There was no evidence of any significant association between markers of inflammation and the neuropathological features of DLB. This finding suggests a limited role for microglia in the response to protein deposition in DLB and may imply that the extent of microglial activation in DLB is independent of protein accumulation. It does not, however, exclude a significant role for microglia in neurodegeneration in DLB.

One possible explanation for this may be related to the pattern of protein deposition in DLB. The disease is characterised by intracellular accumulations of α-syn in the form of LRP, along with pre-synaptic deposits of α-syn. Although it is theoretically possible for intracellular components of α-syn to be presented on antigen presenting cells, this is unlikely to happen until neurons are already degenerating. Thus the extent of inflammation in the DLB brain may not correlate with the extent of neuropathology since much of that neuropathology is not immediately available to interact with immune cells. In addition, microglial activation in DLB may be more pronounced in cases with significant neurodegeneration, which of course may be caused by a process different than protein accumulation.

### 4.3.10 Comparison with AD study

The AD study showed significantly higher expression of CD68, CD64 and CD16 in AD, along with a lower CD32b load, compared with matched controls (Dr Sonja Rakic, unpublished). The higher levels of markers associated with a phagocytic phenotype, along with the reduction in an inhibitory FcγR, suggests a strong swing towards a phagocytic immunophenotype of microglia in AD. In support of this, previous work has shown a positive correlation between AD pathology and microglial proteins associated with phagocytosis in the form of CD68 and "macrophage scavenger receptor-A" (MSR-A) [174]. Additionally, that study showed that those markers of microglial phagocytosis (MSR-A and CD68) were positively associated with the presence of dementia and poorer cognitive function.

Interestingly, the expression of both anti-inflammatory markers (IL4 and CHI3L1) were unchanged in DLB compared with controls. Both of these markers seem to be associated with an immunosuppressive environment, and were in fact found to be elevated in AD compared to controls (Dr Sonja Rakic, unpublished), possibly indicating a stronger anti-inflammatory profile in the AD brain compared with DLB. It may be that since there is a more pronounced phagocytic phenotype of microglia in AD, an associated anti-inflammatory reaction is required in an attempt to maintain an equilibrium and prevent bystander damage of neurons and synapses.

Perhaps most strikingly, there appears to be a unique FcγR profile in DLB, with a largely unchanged balance of antibody-mediated microglial activation compared with the more pronounced phagocytic phenotype found in AD. This suggests that there may be contrasting levels of stimulation of microglial cells by IgG and/or immune complexes in the brains of patients with DLB and AD. There are a number of possible theories as to why this might be the case. One possible cause for this could be reduced presence of IgG or immune complexes in the DLB brain, possibly reflecting differences in systemic immunity between these two diseases, with reduced infiltration of peripheral IgG across the BBB in DLB. Another hypothesis could be related to the relative paucity of Aβ plaque pathology in DLB compared to AD, with a possible associated reduction of IgG bound to those plaques leading to less prominent antibody-mediated phagocytosis by microglia in DLB. Although antibody-mediated microglial phagocytosis of Aβ plaques has been described in immunised AD models [399] and IgG has been identified around Aβ plaques [231] in AD, the role of endogenous anti-Aβ IgG in AD is still unclear. Therefore it is not yet possible to be certain if the difference in FcγR profile between DLB and AD is driven by different levels of antibody-mediated clearance of Aβ plaques between the two diseases.

A further possible reason for the discrepancy in FcγR profile between DLB and AD may be related to the different extent of cortical atrophy seen in the two diseases. Structural brain imaging has shown that cortical atrophy is significantly less prominent in DLB than it is in AD [77], suggesting that the level of neuronal loss and damage to tissue micro-architecture may be less severe in DLB. This may indeed be associated with less prominent antibody-mediated phagocytosis by microglia in DLB, either due to milder bystander damage to healthy brain tissue or because microglia in DLB do not respond to neurodegeneration.

It is notable that while there were no significant associations between markers of neuroinflammation and neuropathology in DLB, examination of the same in AD showed numerous statistically significant positive associations. Aβ positively correlated with HLA-DR, CD64, CD32a, CD32b and CD16; while ptau positively correlated with CD68, CD64 and IL4R. The AD group also showed numerous positive associations between different markers of inflammation,

including within the classical markers of microglial activation and within the FcγR. This, perhaps surprisingly, demonstrates a high level of coordination between microglial phenotypes in AD. One explanation could be that the extent of neuropathology and neurodegeneration in AD is associated with widespread activation of microglia, which are adopting a range of phenotypes at once in an attempt to combat protein deposition as well as responding to synaptic and neuronal loss. This includes increased expression of anti-inflammatory markers in AD, possibly occurring as a mechanism by which the cerebral immune system attempts to counter neurodegeneration by ameliorating further tissue damage. In support of this, previous work has shown evidence of an immunosuppressive environment in AD [395, 400], suggesting that alternative activation of microglia may play a key role in cerebral inflammation in AD.

That AD and DLB are both characterised by intracellular and extracellular cerebral protein deposition but differ so dramatically in terms of associations of neuropathology with markers of inflammation, indicates that they are caused by different mechanisms of neurodegeneration. The process of protein deposition in DLB may indeed be independent of neuroinflammation, and there may also be a lack of immune response to protein deposition in DLB.

### 4.3.11 Strengths and limitations

One of the major strengths of this study is the use of brain tissue to the examine microglial phenotype in human DLB, rather than in an experimental or pre-clinical model of the disease, which are reliant on artificial expression of pathology. The use of human tissue allows investigation of microglial phenotype in the context of complex human physiology and disease processes. However, use of this type of tissue does have some limitations. Antigen detection in post-mortem brain tissue is prone to problems when there are delays in fixation and processing of the brain following death [166]. This potential confounder was mitigated in CIDL through the use of brain tissue obtained from recognised UK brain banks, which guarantees a level of standardisation of tissue processing. Brain banks only provided cases where there was <72 hours delay from death to post-mortem, thereby minimising any bias from delayed fixation.

The large sample size examined in CIDL is a significant strength of the study, and sets it apart from previous literature in this area. The cohort of 59 post-mortem cases, including 30 DLB cases, is the largest by far of any similar study examining microglial activation in DLB. Furthermore, the use of a range of markers to detect functional phenotypes of microglia in DLB is unique to CIDL as previous studies in this area have examined just one or two markers. Furthermore, the use of classical microglial activation markers in CIDL allows for comparisons with data from other groups [401]. However, some concerns have been raised about the method of immunophenotyping microglia using markers such as HLA-DR, due to their widespread expression in the CNS and variability due to age, gender, fixation and ethnicity [171, 402]. To prevent the effects of these confounders, the variables of age, gender and length of fixation were controlled for in the design of CIDL by ensuring that the DLB and control groups were well matched.

The use of percent protein load on immunostained tissue to measure the burden of protein accumulation is a strength because it is well validated, has been used extensively in published work, and is an objective method which removes significant bias when assessing tissue. It does not, however, provide information about number of pathological entities (e.g. plaques, tangles, LB) or cells (e.g. microglia), nor detail regarding gene expression. Other techniques, such as polymerase chain reaction (PCR), could be used to examine the latter. Resource constraints meant that it was decided that one methodology would be used to examine a large number of cases and markers.

Case selection for CIDL was partly dependent on semi-quantitative assessment of neuropathology, specifically Braak ptau stage. However, there are potential limitations to this approach. The extent of ptau pathology has been shown to differ significantly in AD cases that have been classified as Braak stage 6 [403], suggesting that semi-quantitative staging of pathology may lead to loss of more detailed information regarding heterogeneity. A further relevant point here relates to the semi-quantitative method used for quantifying α-syn in CIDL. One of five scores were assigned to all cases to represent the stage of severity of LRP. This method was used because quantification of protein load would very likely have missed most LRP, as this is distributed relatively sparsely even in severe disease. The semi-quantitative method of measuring

LRP may have masked significant variations in severity of pathology within each of the five scores. This may have prevented the accurate representation of pathological phenotype of DLB cases and impeded the detection of significant correlations between LRP and markers of inflammation. It is also noteworthy that immunostaining for α-syn revealed variable levels of background staining and "synaptophysin-like immunoreactivity", a finding which has been reported before [393, 404]. The use of protein load as a measurement of LRP could have led to the inaccurate measurement of synaptic staining as LRP in some control cases. Therefore I am confident that the use of semi-quantitative assessment of LRP, as recommended by international consensus guidance in DLB [9], was the most appropriate method to be used.

Consistency of sampling of cases was ensured by examining the same area of the brain in all cases. The superior temporal sulcus was marked by an experienced neuropathologist and all markers were quantified in the same area of interest. It has been previously shown that the degree of Aβ pathology differs between gyri and sulci [405], and therefore the consistent sampling of the same sulcus prevented this from being a confounder. The inclusion of just one neocortical area could be considered a limitation. Evidence from PD studies have shown that LRP may progress sequentially from the midbrain to the dorsal forebrain and on to the neocortex [63]. However, this pattern may be markedly different to mixed DLB and AD cases [64]. The presence of a certain level of neuropathology in one part of the neocortex may not represent the severity of overall neuropathology in that case, but instead the stage of spread of pathology. Related to this, the extent and location of microglial activation may be altered in different brain regions. An alternative methodology to CIDL would have been to study several cortical and sub-cortical areas, perhaps focussing on areas of most severe α-syn burden. During the planning phase of the project a decision was made to study one brain area with a large number of markers, primarily due to limited resources.

Examining the brain after death as part of a retrospective observational study means that is only possible to assess inflammation at the terminal stage of disease. Whilst important conclusions can be drawn from data at this time point, it is not possible to exclude the possibility of a different inflammatory profile being present in mild disease that is undetectable in the terminal

stages. It is also problematic to deduce causality, but of course there are currently no ethical methods in obtaining brain tissue of dementia cases during life, in sufficient numbers. Imaging studies of *in-vivo* inflammatory markers are a method of examining neuroinflammation during life but, as previously discussed, there are issues relating to the validity of the markers currently used.

Overall, the novelty of this study is in the use of a number of different markers of microglial phenotype in a large number of cases of DLB, in a research field where there is little previous literature.

### 4.3.12 Summary

A summary of the microglial immunophenotype detected in DLB and AD is shown in table 4.18. Results shaded in green demonstrate a decrease in protein load for that marker, while results shaded in red demonstrate an increase in protein load. Unshaded results demonstrate no change in protein load for that marker.

**Table 4.18: Changes in inflammatory markers in DLB and AD**

| Marker | Description | DLB | AD |
|---|---|---|---|
| **Iba1** | Motility | ↔ | ↔ |
| **HLA-DR** | Antigen presentation | ↔ | ↔ |
| **CD68** | Phagocytosis | ↔ | ↑ |
| **CD64** | Activating FcγR | ↔ | ↑ |
| **CD32a** | Activating FcγR | ↓ | ↔ |
| **CD32b** | Inhibitory FcγR | ↔ | ↓ |
| **CD16** | Activating FcγR | ↑ | ↑ |
| **IL4R** | Anti-inflammatory | ↔ | ↑ |
| **CHI3L1** | Anti-inflammatory | ↔ | ↑ |

Grey = No change in protein load
Green = Decrease in protein load
Red = Increase in protein load
DLB data from CIDL study
AD data from Dr Sonja Rakic, unpublished

The role of microglia in DLB has not been conclusively outlined to date, with previous literature reporting conflicting results. The overall finding of CIDL is that DLB is characterised by a distinct lack of microglial activation of any phenotype. This is particularly striking when compared with data from an associated study in AD, which showed a pronounced phagocytic phenotype of microglia. The difference in microglial phenotypes between the two diseases may be driven by a number of factors, including higher levels of Aβ and ptau pathology in AD than in DLB.

There is much epidemiological and genetic evidence supporting a role for inflammation in the aetiology of AD, as well as post-mortem evidence that microglial activation occurs as a consequence of protein deposition in the disease. In contrast, the only GWAS in DLB to date did not find any significant loci related to inflammation, and much of the previous post-mortem literature has not found a significant increase in microglial activation in this disease. Data from *in-vivo* imaging studies do appear to show a role for increased cerebral inflammation in DLB, specifically early in the disease process. Data from CIDL supports the hypothesis that neuropathology in DLB is not associated with significant microglial activation. Therefore, overall it appears that inflammation does not increase the risk of DLB, nor does it respond to extracellular protein deposition in the disease. Hence there may be two strikingly different immunopathogenic disease processes occurring in DLB and AD.

Potential links between the two studies presented in chapters 3 and 4, which separately investigated peripheral and cerebral inflammation in DLB, are particularly thought-provoking and will be discussed further in chapter 5.

# Chapter 5: Discussion and future work

## 5.1 Discussion

The role of inflammation in DLB has not been well researched to date. This project aimed to investigate this area through a clinical study investigating peripheral inflammation and a post-mortem study investigating cerebral inflammation in DLB. The analysis of both peripheral and cerebral inflammation allowed a unique opportunity to examine the immunophenotype of DLB from two diverse, but related, perspectives. Overall, changes in inflammation in the periphery and brain were discovered, some of which may be linked.

In the brain, subtle changes to the FcγR profile were found in DLB, with the overall balance of activation appearing unchanged. This implies a lack of significant role for antibody-mediated microglial activation in DLB. This finding contrasts with the profile of FcγR detected in the AD brain, where the balance appeared to be heavily tipped towards an activated profile. This contrasting picture of differential levels of antibody-mediated microglial activation between the two diseases suggests that they may be characterised by different levels of antibody in the brain. FcγR are known to bind to IgG (a common class of antibody), which is normally only present in the periphery. However, murine studies have shown that up to 0.1% of systemic IgG does indeed cross the BBB into the cerebral cortex [183, 184], and is mainly found in diffuse patterns surrounding blood vessels [406]. The extent of IgG crossing the BBB has been shown to increase when the BBB is leaky, secondary to age or vascular disease [407, 408], or indeed due to systemic inflammation [200]. Furthermore, breakdown of the BBB has been extensively established in AD and in animal models of the disease [409]. Therefore the increase in antibody-mediated microglial activation in AD may be driven by degeneration of the BBB, allowing influx of IgG from the periphery into the brain and subsequently activating microglia via FcγR. This process appears to be less prominent in DLB compared with AD.

It is significant to note that FcγR bound by immune complexes have previously been shown to cause increased microglial activation and neuronal damage in

humans [410]. Such immune complexes in the brain could include antibody-coated micro-organisms but are much more likely to consist of antibody bound to protein deposits or components of cell debris. Indeed autoantibodies have been found in AD and could enter the brain causing microglial activation [411]. The lack of an activated FcγR profile in the DLB brain is consistent with the absence of microglial activation found in CIDL, as measured by the levels of classical markers of activation (Iba1, HLA-DR and CD68). This supports the idea that cerebral inflammation does not play a major role in DLB, at least in the terminal stages of the disease, possibly secondary to a relative lack of neuropathology or cerebral atrophy in DLB compared with AD. The lack of associations between neuroinflammation and neuropathology certainly supports the hypothesis that neuroinflammation does not play a prominent role in the response to extracellular protein deposition in DLB. It could be that synaptic or neurochemical changes may be more significant in the pathogenesis and symptomatology of DLB, with these factors certainly being supported by the fluctuations in cognition that occur in DLB.

Peripherally in DLB a downregulated adaptive immune system was identified, as demonstrated by reduced activation of B cells and a lower proportion of helper T cells. This immune profile indicates senescence of the adaptive immune system in DLB, possibly secondary to chronic activation causing an exhausted phenotype. One potential cause for this chronic activation is repeated trauma and/or infection, both of which are more common in DLB than in AD.

The reduction in cells associated with activation of the adaptive immune system in DLB may be linked with the data from serum cytokine analysis. Serum concentrations of IL1β and IL6 were elevated in DLB compared with controls. These cytokines are classically released acutely in response to innate immune system activation, typically in response to infections. A healthy adaptive immune system would normally modulate the innate immune response, thus preventing chronic pro-inflammatory cytokine release. Therefore, it may be that there is a diminished adaptive immune response to acute inflammatory events in DLB, possibly secondary to immunosenescence caused by chronic antigen exposure. This could lead to inhibited negative feedback that fails to downregulate the innate immune response, causing

chronic elevation of pro-inflammatory cytokines in the periphery in DLB. The absence of increased levels of cytokines typically associated with chronic humoral and cell-mediated inflammation, such as IL4, IL10, IL12, IL13 [412], demonstrates that chronic peripheral inflammation may not play a significant role in DLB. There may be some form of chronic acute-phase reaction found in DLB, occurring due to a lack of adaptive immune switch-off of the innate immune response. This lack of adaptive immune response in DLB may also help to explain the absence of significant neuroinflammation found in DLB, as will be discussed below.

One of the roles of activated B cells is in the production of IgG following differentiation into plasma cells. If in DLB the adaptive immune response is impaired, there may be a relative lack of specific response to pathogens, leading to impaired peripheral IgG release. This has a number of implications. Firstly, this could predispose to higher rates of infection in DLB, or even contribute to a chronic innate immune state where there is a persistent low-grade innate immune response. The latter of these two points could explain the delirium-like clinical presentation of DLB, with fluctuations and visual hallucinations. If it is proven that there is less extensive propensity of the BBB to leak IgG in DLB, the lower level of peripheral IgG may indeed lead to very limited influx into the brain. This could be associated with reduced FcγR activation in the brain, with diminished phagocytic activity, less bystander damage and potentially less cerebral atrophy. This hypothesis would certainly be supported by data from CIDL showing an absence of microglial phenotype associated with antibody-mediated phagocytosis.

One particularly relevant previous study has highlighted the potential involvement of the peripheral adaptive immune system in PD neurodegeneration. Orr et al. found increased IgG positive neurons in the substantia nigra in PD, with the number positively correlating with the number of HLA-DR positive microglia in the same brain region. There was also increased expression of CD64 (FcγRI) on surrounding microglia, which were shown to contain pigment granules consistent with completed phagocytosis of the IgG-positive pigmented neurons. Furthermore, serum from these patients contained IgG that specifically bound to neurons in the substantia nigra [335]. The potential involvement of IgG in a localised region of the brain, and indeed

an area which is consistent with PD pathology, supports a role for the adaptive immune system in the pathogenesis of PD.

In support of the above hypothesis for a role of adaptive immunity in targeting cerebral pathology, there have been numerous clinical studies trialling both passive and active immunotherapy targeting Aβ in AD. These typically either involve peripheral injections of monoclonal antibodies, or in the case of active immunotherapy, involve peripheral injection of epitopes of Aβ in order to induce an adaptive immune response. Whilst there is evidence in animal models that these strategies are effective, there is limited evidence of any marked clinical benefit in humans to date [413]. However, it is important to note that the method of action for these treatments is the infiltration of specific anti-Aβ antibodies from the periphery into the CNS, resulting in the coating of amyloid plaques with IgG and the promotion of phagocytosis by microglia, modulated by FcγR. Indeed, increased expression of FcγR has been described in AD previously [230], with further work showing serum-derived IgG from AD patients causing selective reduction of cholinergic neurons when injected into rodent brains [414].

The paragraphs above support the hypothesis that peripheral auto-antibodies may play a role in neurodegeneration in AD and PD. Antibody-mediated phagocytosis in dementia is likely to be modulated by FcγR, and occurs as a result of activation through a number of potential routes. These include activation by IgG-coated neurons or extracellular pathology, with the source of antibody likely to be from outside the CNS. If these peripheral antibodies are antigen specific, against for example Aβ or neuronal agents, the IgG may be retained in the brain and have a higher propensity to provoke antibody-mediated microglial activation. If indeed the absence of antibody-mediated microglial activation is a feature of DLB, and this is possibly driven by a lack of adaptive immune system signal from the periphery, it would be interesting to contrast this hypothesis to AD.

The findings in the AD study examining cerebral inflammation (Dr Sonja Rakic, unpublished) and from the AD arm of SILAD, are certainly noteworthy and appear to show an opposing picture to that found in DLB. B cell activation and the proportion of helper T cells was found to be higher in AD than controls and statistically significantly higher than in DLB, suggesting a divergent profile

between AD and DLB. Clinical studies have shown varying levels of peripheral cytokines associated with innate immunity in AD [235], while epidemiological studies have shown roles for a number of chronic inflammatory diseases in increasing the risk of developing sporadic AD [213]. In addition, peripheral lymphocytes in AD have been previously shown to switch towards a memory phenotype, indicating that there may be a persistent antigenic challenge in AD, possibly causing chronic activation of the adaptive immune system [249]. The activation of the innate immune response in AD may well trigger an exaggerated adaptive immune response, with increased proportions of helper T cells and increased activation of B cells found in SILAD, perhaps contributing to increased peripheral IgG. AD is also known to be associated with degeneration of the BBB [409], possibly making it more permeable to immune cells and components. Increased influx of IgG into the AD brain could plausibly trigger increased Fc$\gamma$R-mediated phagocytosis, perhaps leading to bystander damage and exaggerating neuroinflammation. This is particularly likely when considering that CD64 is known to be activated by monomeric IgG without the presence of an immune complex [177]. This hypothesis shows that the contrasting findings of cerebral inflammation in DLB and AD may indeed be driven by the differing levels of adaptive immune system activation present in the two diseases, along with potential differences in the permeability of the BBB.

With regards to cytokine ratios following stimulation of PBMC with mitogenic agents, there were no differences found between both dementia groups and controls. However, when stratified by *APOE* genotype, the PBMC of AD patients who were $\varepsilon$4 carriers showed less prominent cytokine production than non-carriers. This contrasted with findings in DLB and controls, where there was no difference between $\varepsilon$4 carriers and non-carriers. These results suggest that the sensitivity of PBMC to antigen and mitogenic challenge is modulated by *APOE* genotype in AD, but not in controls or DLB. That AD $\varepsilon$4 carriers showed decreased IL1$\beta$ and TNF$\alpha$ production, yet there was no such effect in DLB, shows a divergent impact of *APOE* status between the two disease states. The impact of *APOE* genotype on cerebral inflammation was not established in CIDL due to limited data from brain banks. However, its importance as a genetic risk

factor for both AD and DLB supports further research into its role not only in peripheral inflammation but also its impact on cerebral inflammation.

When examined as a whole, the results of this project appear to show a lack of significant cerebral inflammation in DLB, possibly secondary to a diminished adaptive immune stimulus from the periphery. This appears to reduce the likelihood that anti-inflammatory therapy would prove beneficial in DLB. However, as previously discussed, evidence from *in-vivo* imaging studies [314, 315] and a recent study examining peripheral cytokines in MCI-DLB [338], indicate that there may be a role for both peripheral and cerebral inflammation early in the disease process in DLB. Similar to AD, any benefits of anti-inflammatory medications in DLB may be limited to mild stages of the disease, rather than once neurodegeneration has become established. It is even possible that benefits may not be maximised unless medications are trialled at the pre-symptomatic phase of disease, although the likelihood of this being proven in a clinical trial depends on biomarkers being available that could accurately predict conversion to disease.

Overall, significant differences have been observed in cerebral and peripheral inflammation between AD and DLB. Figures 5.1 and 5.2 illustrate the possible mechanism by which the contrasting peripheral inflammatory profiles in AD and DLB may drive cerebral inflammation.

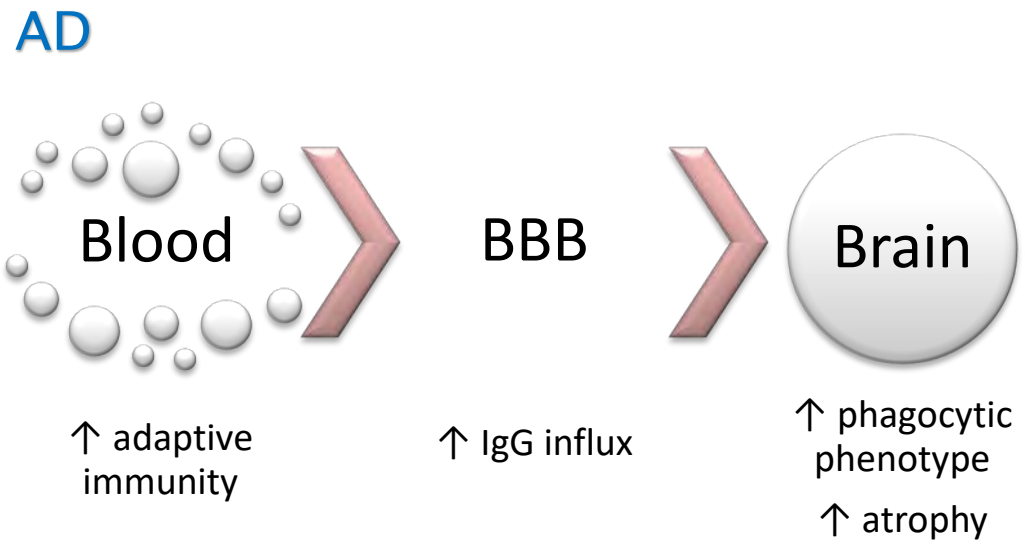



**Figure 5.1: Illustration of possible mechanism of interaction between peripheral and cerebral inflammation in AD**

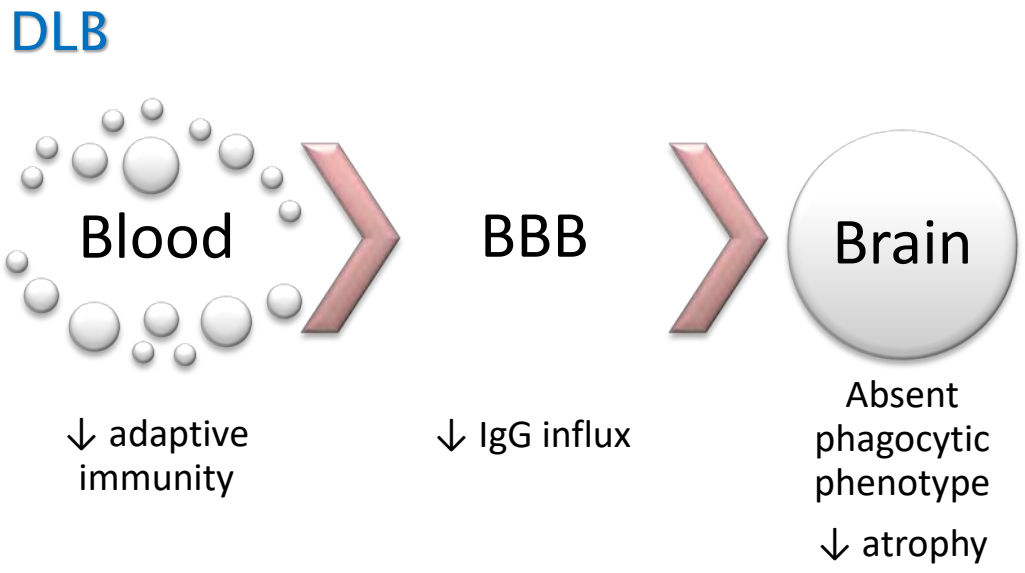



**Figure 5.2: Illustration of possible mechanism of interaction between peripheral and cerebral inflammation in DLB**

The novel findings in these two studies warrant comments about potential mechanisms between them. However, it should be noted that these two studies were not performed on the same cohort of patients, and that they may well be assessing immunophenotype at different stages of the disease process. Although caution should be applied in the over-interpretation of any link between the results of the two studies, the fact that there is very little known about the aetiology of DLB permits speculation about potential immunological mechanisms in the disease. The profile of cerebral and peripheral inflammation in DLB and AD is likely to change during pathogenesis and therefore the above hypothesis needs to be confirmed in future work.

## 5.2 Implications for future work

The identification of changes in the immune system in DLB warrants further investigation in order to develop our understanding of the aetiology and pathogenesis of DLB.

Taking into account the limitations detailed in previous chapters, several recommendations can be made for further work. There is an urgent need to standardise methodologies for studies of peripheral inflammation in order to improve reproducibility of results. This should include standardisation of the time of phlebotomy, speed and type of processing of blood samples and storage procedures. This is essential to minimise the risk of further conflicting results between research groups. Novel results in flow cytometry presented in this project should be further examined to allow confirmation of findings. The examination of not only serum and PBMC data, but combining this with *in-vivo* imaging of brain inflammation and CSF studies would allow a more robust overview of inflammation in DLB.

I propose several recommendations to improve the quality of future work examining peripheral inflammation in dementia. As previously discussed, the flow cytometry panel of markers used in SILAD detected primary PBMC populations. If more PBMC were isolated from each patient, by drawing a larger blood sample, further analysis of immunophenotype could be performed. Several panels of markers could be used to differentiate the subsets of T and B

cells in more detail, which could include markers such as PD1 for lymphocyte exhaustion [195] or intracellular cytokine markers to examine different effector T cell subsets such as Th1 or regulatory T cells. Furthermore, cell sorting could be used to identify cytokine production by individual cell subsets, providing invaluable information regarding the precise phenotypes of different parts of the innate and adaptive immune system. In addition, expression of inflammatory genes could be examined using Paxgene samples, which would further enhance our understanding of the aetiological processes behind DLB.

Future post-mortem work should ideally be extended to look at multiple regions of the brain to investigate whether patterns of inflammation may differ. This may allow for increased detection of correlations with neuropathology. Additionally, examining an AD and DLB group concurrently with a matched control group would allow direct comparisons to be made between the two diseases. Ideally markers of inflammation could be correlated with the duration of disease, but this clinical measure is difficult to precisely quantify. UK brain banks have been increasingly co-ordinating their efforts to allow long-term neuropsychological follow-up of patients with detailed medical histories. The availability of this information would be invaluable when attempting to test for associations between clinical features and cerebral markers of inflammation.

A number of other markers could be assessed when examining cerebral inflammation. The hypothesis detailed above regarding a role for IgG levels in the brain could be confirmed using immunohistochemistry against IgG directly. Examination of IgG in the brain directed against Aβ or α-syn would be particularly interesting and allow an insight into the role of antibody-mediated clearance of neuropathology in AD and DLB. The possible infiltration of B cells and T cells into the human brain in DLB and AD could also be examined using the same methods, as could markers of BBB permeability. Each of these pieces of future work could improve knowledge of the role of the adaptive immune system in the degenerating DLB brain.

The *in-vivo* imaging studies reviewed previously have potential limitations. Both used PK11195 as a ligand for TSPO, a marker expressed on microglia. However, it has also been shown that this ligand also binds to reactive astrocytes in the brain [220], and that there is astrogliosis without microgliosis

in the pulvinar in DLB [332]. This implies that that there may also be a prominent role for astrocytes in the DLB disease process, particularly early in the disease. Potential future work could include immunohistochemistry for GFAP, which is expressed on astrocytes, to investigate their role in the neocortex in DLB.

The role of *APOE* genotype should also be considered in future studies examining inflammation, particularly when performing stimulation studies. Unfortunately, sufficient data on *APOE* status in CIDL was not collected and therefore it was impossible to determine whether this was an important factor in DLB. Brain banks were repeatedly approached for APOE genotype data on cases included in CIDL. The LNDBB in particular fed back that they had contacted all other users of the same brain tissue, but disappointingly did not receive responses from any collaborators for information of *APOE* genotype.

The use of double or triple immunohistochemistry would have allowed visualisation of the interactions between brain pathology and inflammatory markers. However, this was not deemed to be essential in order to answer the aims of the study, and in fact the relationship between neuropathology and microglia in AD and LBD is already well-established. In order to examine microglial phenotype in the DLB brain in more detail, further cortical and subcortical areas could be examined. This would require significantly more time and resource. A technique called tissue micro-array has been used to sample neuropathological burden in several cortical regions [415], and could be used to assess microglial phenotype. Furthermore, the use of stereological analysis of immunostained human brain tissue would allow 3-dimensional visualisation of the interactions between neuropathology and microglia, with more detailed analysis of the density of pathology and microglia.

Ultimately, the growing range of techniques used to identify the role of inflammation leads to the need for larger numbers of more well-defined DLB cases. An ideal study would be one that prospectively collects a cohort of DLB cases, with frequent clinical assessments, blood samples and serial *in-vivo* imaging, followed by examination of the same cases at post-mortem examination. Due to the obvious resource implications of such a study, this will only be possible through international collaboration of research groups and brain banks working together to standardise procedures and share data.

## 5.3 Concluding remarks

DLB is an under-researched yet important cause of dementia. There is now extensive evidence supporting a role for inflammation throughout the pathogenesis of AD and PD, but to date the picture has been much less clear in DLB. Overall, there is little known about the aetiology of DLB.

This project has revealed novel findings in the fields of cerebral and peripheral inflammation in DLB. The primary benefit of the information gained during these studies is to develop our knowledge about the aetiology and progression of DLB. This project has shown that microglial phenotype is only subtly different in DLB compared with controls, and that it differs dramatically from the pronounced phagocytic phenotype of microglia found in AD. This project also revealed the peripheral inflammatory phenotype in DLB, demonstrating increased pro-inflammatory cytokine levels and a reduction in adaptive immunity. The cerebral and peripheral immune profiles in DLB may well be linked, with possible downregulation of antigen presentation to the brain from the periphery explaining a lack of phagocytic phenotype in the cerebrum in DLB.

There are a number of clinical implications that arise from this study. Firstly, it does not appear that cerebral inflammation plays as prominent a role in DLB as it does in AD. Therefore the trialling of anti-inflammatory medication in established DLB is likely to be unhelpful. Furthermore, alterations in peripheral lymphocyte subsets in DLB that are unique from AD holds much promise in the development of blood based biomarkers to differentiate DLB from AD. This is most likely to succeed as part of a larger biomarker panel using cytokine concentrations, PBMC populations and perhaps neuropathological markers in body fluid.

The findings from this project have helped to clarify the immunophenotype of DLB. It is only by improving our understanding of the disease mechanisms of DLB that we can hope to open new avenues for biomarkers and treatments for this disease.

# Appendix A

## A.1 Publications

One paper and three abstracts were published by me during the course of this project. A further two papers are in preparation for submission.

Papers in preparation

- Jay Amin, Robert Dorey, Daisy Williams, Emmanuele Tommasino, Yuri Casal, Charles Dupuy, Clive Holmes, Delphine Boche. Microglial phenotype in Dementia with Lewy bodies.
- Jay Amin, Zoe Clough, Florence Smith, Anthony Williams, Yifang Gao, Lindsey Chudley, Jessica Teeling, Laurie Lau, Delphine Boche, Clive Holmes. Peripheral inflammation in Dementia with Lewy bodies: a cross-sectional clinical study.

Conference contributions

- Jay Amin, Anthony Williams, Jessica Teeling, Robert Dorey, Daisy Williams, Emmanuele Tommasino, Delphine Boche, Clive Holmes. Central and systemic inflammation in Dementia with Lewy bodies and Alzheimer's disease [abstract]. Neuropathology and Applied Neurobiology 2017; 43(supplementary issue 1):12-13
- Jay Amin, Anthony Williams, Jessica Teeling, Delphine Boche, Clive Holmes. Central and systemic inflammation in Dementia with Lewy bodies and Alzheimer's disease [abstract]. Neuropathology and Applied Neurobiology 2016; 42(supplementary issue 1):36
- Jay Amin, Anthony Williams, Jessica Teeling, Delphine Boche, Clive Holmes Central and systemic inflammation in Dementia with Lewy bodies and Alzheimer's disease [abstract]. American Journal of Neurodegenerative Disease 2015; 4(supplementary issue 1):64. www.ajnd.us/ISSN:2165-591X/2015 International DLB Conf.

Reviews

- Jay Amin, Delphine Boche, Sonja Rakic, What do we know about the inflammasome in humans? Brain Pathology 2017; 27(2): 192-204.

## A.2 Presentations

I have presented Information from this project at the following conferences and meetings, listed in reverse date order.

| Name of meeting/conference | Date | Presentation |
|---|---|---|
| Southampton Neuroscience Group (SoNG) seminar, University of Southampton | 13/09/18 | Oral (Invited) |
| Memory Assessment and Research Centre update, Southern Health NHS Foundation Trust | 21/06/18 | Oral |
| Alzheimer's disease/Parkinson's disease (AD/PD) 2018 Conference, Torino, Italy | 18/03/18 | Oral |
| Alzheimer's Association International Conference, London | 17/07/17 | Oral |
| Southampton Medical and Health Research Conference, Southampton | 15/06/17 | Poster and Oral (Prize winner) |
| RcPsych Faculty of Old Age Annual Scientific meeting, Bristol | 24/03/17 | Oral (Prize winner) |
| Alzheimer's Research UK annual conference, Manchester | 14/03/17 | Poster |
| British Neuropathological Society Annual Meeting, London | 01/03/17 | Oral |
| Lewy Body Society meeting, Centre for Life, Newcastle University | 17/11/16 | Oral (Invited) |
| Faculty of Medicine conference, University of Southampton | 23/06/16 | Oral (Prize winner) |
| The 2016 Alzheimer's Disease congress, O2 London | 07/06/16 | Oral (Invited) |
| Alzheimer's Research UK annual conference, Manchester | 07/03/16 | Poster |
| British Neuropathological Society conference, London | 02/03/16 | Poster |
| Southampton Neuroscience Group (SoNG) seminar, University of Southampton | 11/02/16 | Oral |
| International Dementia with Lewy Bodies conference, FL, USA | 30/11/15 | Poster |
| Southampton Neuroscience Group (SoNG) conference, University of Southampton | 17/09/15 | Poster |
| Venusberg Neuroinflammation conference, Bonn, Germany | 06/05/15 | Poster |
| Dementia Action Research & Education (DARE) network, University of Southampton | 27/11/14 | Oral |
| Alzheimer's Research UK conference 2015, London | 10/03/14 | Poster |

# Appendix B

The following pages consist of study material related to the clinical study (SILAD) described in Chapter 3, as listed below.

- Approval letter from National Research Ethics Service Committee London Hampstead (reference 14/LO/1510). 4 pages.
- Participant information sheet was provided to participants in order for them to make an informed decision regarding their involvement in the study. 8 pages.
- Consent forms were signed by participants and their study partners at their single visit, following an assessment of capacity to make this decision. 4 pages.
- Source sheet was used by the clinician to record all details relating to the study visit, including demographic details and medical/drug history. 2 pages.

NHS
Health Research Authority
National Research Ethics Service
NRES Committee London – Hampstead
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7434

18 August 2014

Professor Clive Holmes
M.A.R.C. Moorgreen
Botley Road
Southampton
SO30 3JB

Dear Professor Holmes

| | |
|---|---|
| **Study title:** | **Systemic inflammation in Dementia with Lewy Bodies and Alzheimer's Disease** |
| **REC reference:** | **14/LO/1510** |
| **IRAS project ID:** | **161251** |

The Proportionate Review Sub-committee of the NRES Committee London - Hampstead reviewed the above application on 13 August 2014.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager Anna Bannister, nrescommittee.london-hampstead@nhs.net

**Ethical opinion**

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

**Conditions of the favourable opinion**

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

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

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

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

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

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

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

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

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

**Ethical review of research sites**

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

**Approved documents**

The documents reviewed and approved were:

| *Document* | *Version* | *Date* |
|---|---|---|
| Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Letter of insurance from sponsor] | v1.0 | 29 July 2014 |
| GP/consultant information sheets or letters [GP information letter] | v1.0 | 25 July 2014 |
| GP/consultant information sheets or letters [GP information letter] | v1.0 | 25 July 2014 |
| IRAS Checklist XML [Checklist_01082014] | | 01 August 2014 |
| Participant consent form [Control Consent Form] | 1.0 | 16 July 2014 |
| Participant consent form [Patient Consent form] | v1.0 | 16 July 2014 |
| Participant consent form [Partner Consent Form] | 1.0 | 16 July 2014 |
| Participant information sheet (PIS) [Participant information sheet] | v1.0 | 16 July 2014 |
| REC Application Form [REC_Form_01082014] | | 01 August 2014 |
| Research protocol or project proposal [Protocol SILADv1.0] | v1.0 | 16 July 2014 |
| Summary CV for Chief Investigator (CI) [Chief investigator CV] | 1.0 | 25 July 2014 |
| Summary CV for supervisor (student research) [Student CV] | 1.0 | 25 July 2014 |

**Membership of the Proportionate Review Sub-Committee**

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

**Statement of compliance**

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

**After ethical review**

Reporting requirements

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

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

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

**User Feedback**

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

**HRA Training**

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

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

| **14/LO/1510** | **Please quote this number on all correspondence** |
|---|---|

Yours sincerely

**Stephanie Ellis**
**Chair**
Email: nrescommittee.london-hampstead@nhs.net

*Enclosures:* *List of names and professions of members who took part in the review*
*"After ethical review – guidance for researchers"*

*Copy to:* *Ms Barbara Halliday*
*Ms Penny Bartlett, Southern Health NHS Foundation Trust*

**NRES Committee London - Hampstead**

**Attendance at PRS Sub-Committee by correspondence**

**Committee Members:**

| *Name* | *Profession* | *Present* | *Notes* |
|---|---|---|---|
| Miss Stephanie Ellis | Former Civil Servant | Yes | |
| Mr Paul Hardiman | Consultant Gynaecologist/Senior Lecturer in Obstetrics and Gynaecology | Yes | |
| Mrs Arlene Renee Seaton | Lay Member | Yes | |

**Also in attendance:**

| *Name* | *Position (or reason for attending)* |
|---|---|
| Miss Anna Bannister | REC Manager |

UNIVERSITY OF Southampton School of Medicine

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www.southernhealth.nhs.uk

# Patient and Study Partner Information Sheet v1.0

**NRES Committee London Hampstead – Ref 14/LO/1510**

**PROTOCOL NUMBER: SILADv1.0**

**Sponsor:** University of Southampton **Principal Investigator:** Clive Holmes

**Participant Initials:** **Participant Number:**

**Study title:** Systemic inflammation in Dementia with Lewy Bodies and Alzheimer's Disease (SILAD)

**Lay title:** The role of the immune system in people with Lewy Body Dementia and Alzheimer's Disease

## Introduction

You are being invited to take part in a research study being conducted at the Memory Assessment and Research Centre.

Participation in this study is voluntary. This means you are free to decide whether or not to take part in this research study.

You may need someone (such as a relative or close friend) to participate in this study with you. We will call them your 'study partner' in this information sheet.

Before you decide to take part, it is important that you understand why the research is being done and what it will involve. This form describes:

• The known possible risks and benefits of participating in this study.

• The procedures you will be asked to complete if you take part in the study.

• The study responsibilities for you and your study partner.

• The choices for your care if you decide not to be in the study.

Please take your time to read the following information. Discuss it with your friends and family if you wish.

If you wish to be in the study then you and your study partner will be asked to sign and date the Patient and Study Partner Informed Consent Form. A copy of the signed Informed Consent Form will be provided to you.

Thank you for taking the time to read this information. Please ask the study staff any questions you may have.

## Who is organising and funding the study?

The study is being organised by Professor Clive Holmes at the Memory Assessment and Research Centre. Tel: 023 8047 5206.

The University of Southampton is the sponsor for this study. The study is funded by Alzheimer's Research UK.

## What is the purpose of the study?

The purpose of the study is to find out more information about the role of the immune system in people diagnosed with Lewy Body Dementia and Alzheimer's Disease and how it may differ from people without a memory disorder.

Many of the symptoms of Lewy Body Dementia and Alzheimer's disease also occur when people suffer with delirium, a condition which some elderly people get when they have an infection. The immune system in Alzheimer's Disease and Lewy Body Dementia has not been well researched but we think that the condition is caused, or made worse, by an overactive immune system which makes it look like an infection is taking place.

We want to see if this is the case and if we can prove it we will be in a much better position to develop new treatments. We also think our research could help allow us to develop a blood test for the disease that means Doctors can diagnose the conditions earlier.

## Why have I been chosen?

Study staff have invited you to participate in this study because you have been diagnosed with either Dementia with Lewy Bodies or Alzheimer's disease, or because you do not have a diagnosis of Dementia.

By participating in this study, you may help create knowledge that could help improve the diagnosis and treatment for people with Dementia with Lewy Bodies and Alzheimer's disease in the future, but this cannot be guaranteed.

120 volunteers will participate in this study which will be carried out at the Memory Assessment and Research Centre Memory Assessment and Research Centre, Tom Rudd Unit, Moorgreen, Botley Road, West End, Southampton, SO30 3JB, United Kingdom.

## What are my responsibilities while I am in the study?

- We ask that you please keep your one-off study appointment and have your study partner attend with you if needed. If you cannot keep your appointment please contact us to reschedule.
- Please allow the trained study researcher or doctor to carry out a blood test at the time of your visit.
- Please tell us if you change your mind about staying in the study.
- Please feel free to ask us any questions you think about.

Whilst you are in this study we ask that you do not take part in any other research study. This is because other studies may affect this study.

## What are the responsibilities of the study partner in the study?

Your study partner is a close friend or relative who knows you well or is the main person that helps you with your daily activities. If you have been diagnosed with Alzheimer's Disease or Dementia with Lewy Bodies we ask that this person is present at the study appointment as your study partner's information about you is an important part of the study.

- They will be asked to sign a consent form for their own involvement with the study.
- They will be asked questions about your symptoms and about your past medical history.
- They will be asked to inform us if you are unwell, or develop new symptoms, if you are unable to let us know yourself.

## What will happen to me if I take part?

If you decide to participate, study staff will ask to visit you in your own home or at the Memory Assessment and Research Centre for a single visit. A brief description of what will happen to you at the visit is reported below.

**Single visit**
Study staff will ask to visit you in your own home or if you prefer at the Memory Assessment and Research Centre. At this visit you will be provided with information about what the study entails. If you and your study partner are still willing to take part, you will be asked to give consent for participating in the study. The study staff will then ask you a series of questions about your health and what medications you take.

You will be asked to complete a range of tests to assess your memory and thinking ability. You will also be asked to complete some brief questionnaires that ask questions about your mood.

The study staff will ask to carry out a blood test with you (30.5ml, which is approximately 2 tablespoons of blood).

Your study partner will be asked to perform some assessments. These assessments will measure your mood and level of thinking ability.

The whole visit will take approximately 2-3 hours to complete.

## Biological samples

At the one-off study visit, taking place at your home or the study centre if you prefer, the research staff will ask to carry out a blood test with you. A total of 30.5ml (which is approximately 2 tablespoons) of blood will be taken. Usually all blood samples are taken at the same time, which means you should not get an extra needle prick.

With your consent we would like to perform a blood test to look at possible genetic risk factors that may be related to memory impairment. We want to find out if genes affect your memory. This genetic information about you will stay private and confidential and will not be given to you or your doctor.

We would also like to use the blood samples to investigate the blood cells that work as part of your immune system. We want to test these immune cells in laboratory experiments to research the way they react to certain conditions. We want to find out if the immune cells in people with your condition react in a common way.

## What will happen to any samples that I give?

Blood samples will be analyzed by the local laboratory at Southampton General Hospital. The samples will be stored in a safe location and will not be labeled with any information that would identify you directly. A participant number that is linked to your information will be used instead.

The University of Southampton will ask for your permission to store some samples of blood for possible future research related to Dementia with Lewy Bodies and Alzheimer's disease. These stored samples will keep your patient identification number and will be stored for a maximum of 8 years after the end of the study. Any sample remaining at that time will be destroyed safely and securely.

If you decide to withdraw from the study for any reason, you can request that the samples you have given are destroyed safely and securely.

## Early withdrawal from the study

The entire study could be discontinued at any time by the following entities: study doctors, the Ethics Committee or the Southern Health NHS Foundation Trust if the safety of research participants is found to be at too much risk.

## What will happen if I don't want to carry on with the study?

You may decide to stop participating in the study at any time without giving any reason. A decision to withdraw will not affect the care you receive. If you decide to withdraw, please tell the study staff. The study staff will discuss with you the best way to stop your participation in this study.

Your study doctor or the study sponsor may take you out of the study if they think it is in your best interests or if you do not follow the study instructions.

## Do I have to take part?

It is up to you to decide if you want to take part in this study or not.

- If you **do** decide to take part, we will give you a copy of this information sheet and we will ask you to sign a consent form.
- If you decide **not** to take part, it will not make any difference to your present or future medical care.
- You are still free to **change your mind later** and withdraw from the study at any time without giving a reason. Again, it will not make any difference to your present or future medical care.

If you decide to participate, you will be told of any important new information that is learned during the course of this research study that might affect your condition or your willingness to remain in the study.

## Your ability to consent

It is important that you are able to give informed consent in order to participate in this study. By this we mean that you fully understand what the study is about and what will happen to you during the study whilst you are taking part. If your condition deteriorates and you are no longer able to give informed consent during the study, you and your study partner's participation will be stopped immediately with no further study procedures carried out.

## What are the possible risks / discomforts of the procedures or tests?

During your study visit, research staff will ask to take a blood sample from you. The risks of drawing blood include temporary discomfort from the needle in your

arm, bruising, swelling at the needle site, and, in rare instances, infection. You may also experience nervousness, tiredness or boredom during the mental testing at your visit.

You are free to stop any test or procedure at any time.

Please report immediately any unusual symptom you may experience during the course of the study to the study staff.

## What are the possible benefits of taking part?

This study may not have a direct benefit for you, but you may feel satisfaction in knowing that your participation in this study may help create knowledge that could help improve the diagnosis and treatment for people with Dementia with Lewy Bodies and Alzheimer's disease in the future, but this cannot be guaranteed.

## What if something goes wrong?

The Southern Health NHS Foundation Trust and the University of Southampton provides insurance in case you are injured or become ill as a result of taking part in this study. If you think you have become hurt or sick as a direct result from the study, please contact the Memory Assessment and Research Centre on 023 8047 5206.

In the event that your participation in this study results in a medical problem your doctor will explain the treatment options available and where you can go to get information and be treated.

If you are not happy with the general care and treatment you receive during the study, please speak first to the study staff, who will try to resolve the problem. They will also tell you about the research clinic's standard complaints procedure in case you wish to take the matter further. Further information on your rights as a research participant and on the complaints procedure can also be provided by Southern Health NHS Foundation Trust Complaints and PALs Team. Address: FREEPOST RSJL-JXSX-ATUE, Complaints and PALS Team, 5 Sterne Road, Tatchbury Mount, Calmore, Southampton, SO40 2RZ, and Tel: 02380 874065.

## What are the alternatives for diagnosis or treatment?

You do not have to take part in this study to receive the NHS standard care available for patients diagnosed Dementia with Lewy Bodies or Alzheimer's disease.

Your participation in this study is voluntary and does not affect your rights or the care given to you. If you choose not to participate in this study, or if you withdraw your consent at any time throughout the study, you will continue to receive care for your condition as usual.

## Who has reviewed the study?

All research in the NHS is reviewed by an independent group of people, called a Research Ethics Committee, to protect your safety, rights, wellbeing and dignity. This study has been considered and given a favourable opinion by the NRES Committee London Hampstead – Ref 14/LO/1510.

## Will my records be kept confidential?

Every effort will be made to keep all information about you private. As far as possible, all of your study records will show only your initials instead of your name. Your medical records will be checked in the clinic and will not be removed from the clinic. To protect the identity of your data, you will be assigned a unique participation number with which your data will be coded in the study database.

If you decide to take part, it will be necessary for qualified members of the NRES Ethics Committee, the sponsor, and applicable regulatory authorities to have access to your medical records to check that the information from the study has been recorded accurately. By signing the consent form, you are giving permission for this to happen. In the event of the study results being sent to regulatory authorities, or published, all your records will be kept confidential, and your name will not be disclosed to anyone outside the clinic. Information that identifies you will be kept confidential unless law requires disclosure. Absolute confidentiality cannot be guaranteed because of the need to provide information as described above.

If you decide to take part in this research study, your authorisation for this study will not expire unless you revoke it. If you do withdraw from this study, the information you have already provided will be kept confidential. It is your right to obtain information on what is recorded about you and request corrections of errors.

With your consent we will notify your General Practitioner (GP) about your participation in this study.

## What will happen to the results of the research study?

The data held for you relating to this study will be accessed by the University of Southampton. We will report the study and the results, submit the results to regulatory authorities, and may publish it in a scientific journal. If the results are published, or are presented at scientific meetings, your identity will not be revealed.

The University of Southampton may combine the health information obtained from participants' study records from this and other research studies. The information will be kept in a database and used for further research purposes. All participant information that is collected from you as a result of your participation in this study will be de-identified (anonymous), which means that you will not be

directly identified and the information cannot be linked to a specific study participant.

## Expenses and payments

There will be no additional cost to you as a result of being in the study. It is important that you understand that you are not being paid to be a participant in this study. However, you will be reimbursed for your costs of being in this study.

**Contact Details:**

If you have any questions about this study or a research-related problem, please contact your study doctor at: Professor Clive Holmes or Dr Jay Amin, Memory Assessment and Research Centre, Tom Rudd Unit, Moorgreen, Botley Road, West End, Southampton, SO30 3JB, Tel: 023 8047 5206

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www.southernhealth.nhs.uk

# Patient and Study Partner Consent Forms v1.00

**NRES Committee London Hampstead – Ref 14/LO/1510**

| | |
|---|---|
| **Full study title:** | Systemic inflammation in Dementia with Lewy Bodies and Alzheimer's Disease |
| **Lay study title:** | The role of the immune system in people with Lewy Body Dementia and Alzheimer's Disease |
| **Study doctor:** | Prof Clive Holmes |
| **Protocol number:** | SILADv1.0 |
| **Patient identification number:** | |

## Study Patient Consent Form

| | | Please initial |
|---|---|---|
| 1 | I confirm I have read and understand the information sheet dated _____ version ____ (or someone has read it to me) for the above study. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I will get a signed copy of this form for my records. | |
| 2 | The study researcher has answered my questions in a way that makes sense to me. I have had time to consider taking part in this study. | |
| 3 | I understand that relevant sections of my medical records and data collected during the study may be looked at by individuals from the sponsor for this study, from UK regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records, providing strict confidentiality is maintained. | |
| 4 | I voluntarily agree to allow study staff to collect, use and share my health data. I understand that I am not giving up any of my legal rights by signing this form. | |
| 5 | I voluntarily agree to allow study staff to collect a blood sample from me. | |
| 6 | I understand that the storage of blood samples for future research is entirely optional. | |

| | | |
|---|---|---|
| 7 | I agree that the blood samples I have given and the information gathered about me can be stored by the University of Southampton for possible use in future research projects. | |
| 8 | I agree for genetic testing to be performed on my blood samples. | |
| 9 | I agree for my study partner to provide information about my mood, behaviour and level of thinking ability | |
| 10 | I agree to complete questionnaires that assess my mood and behaviour. | |
| 11 | I agree to perform tests which assess my memory and thinking ability. | |
| 12 | I understand that I am free to stop any assessment, test or questionnaire at any time. I understand that I do not have to answer study questions or provide a reason to study staff for refusing to answer a question. | |
| 13 | I agree to my GP being informed of my participation in the study. | |
| 14 | I understand that if I am no longer able to fully consent to my involvement in this study that mine and my study partner's participation will be stopped immediately with no further study procedures carried out. | |
| 15 | I agree to take part in the above study. | |

**PATIENT**

Print name: _____

Signature: _____

Date: _____

**INVESTIGATOR**

- I have carefully explained to both the patient and the study partner the nature and purpose of the above study.
- There has been an opportunity for both the patient and the study partner to ask questions about this research study.
- I have answered all questions that the patient and study partner have about this study.

Print name: _____

Signature: _____

Date: _____

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**NRES Committee London Hampstead – Ref 14/LO/1510**

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| **Study doctor:** | Prof Clive Holmes |
| **Protocol number:** | SILADv1.0 |
| **Patient identification number:** | |

## Study Partner Consent Form

| | | Please initial |
|---|---|---|
| 1 | I confirm I have read and understand the information sheet dated _____ version ____ (or someone has read it to me) for the above study. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I will get a signed copy of this form for my records. | |
| 2 | I have had the time to consider taking part, have had the opportunity to ask questions, and these questions have been answered in a way that makes sense to me. | |
| 3 | I confirm that I am in regular contact with the patient. | |
| 4 | I agree to provide information about the patient's mood, behaviour and thinking ability. | |
| 5 | I understand that I am free to stop any assessment or questionnaire at any time. I understand that I do not have to answer study questions or provide a reason to study staff for refusing to answer a question. | |

| | | |
|---|---|---|
| 6 | I understand that if I cannot fulfil the study responsibilities I should let the study staff know. I understand that I may be asked to find someone else to take over these responsibilities for the time that I am unavailable. | |
| 7 | I will endeavour to ensure that the patient will attend the required visit, but will not force the patient to attend. Should the patient become unwilling to attend the study visit I will inform study staff. I will try to accompany the patient at the study visit. | |
| 8 | I understand that if the patient is unable to give ongoing informed consent to participate in the study that mine and the patient's participation will be stopped immediately with no further study procedures carried out. | |
| 9 | I agree to participate in this study, to attend the study visit and to provide information on how the patient is doing. | |

**STUDY PARTNER**

Print name: _____

Signature: _____

Date: _____

**INVESTIGATOR**

- I have carefully explained to both the patient and the study partner the nature and purpose of the above study.
- There has been an opportunity for both the patient and the study partner to ask questions about this research study.
- I have answered all questions that the patient and study partner have about this study.

Print name: _____

Signature: _____

Date: _____

**SINGLE VISIT: DLB subject**

Name ……..…… D.O.B ……. NHS or HOSP no ……..……..
Date …… Time ……..….. Subject number …….
Inclusion/Exclusion criteria reviewed: Yes / No By whom: ……..…….
Diagnosis *(using attached criteria)* ……. Age when diagnosed ……

Informed consent taken: Yes / No By whom: ……..…….
Informed consent *(please provide narrative)*
……
……
……
……
……
Subject received copy of PIS and consents? Yes / No
Study partner received copy of PIS and consents? Yes / No

**Demographics**
Height …… Weight ……
Years of formal education …… Relationship status ..……..……..
Sex: M □ F □ Ethnic origin ……..… Occupation ……..……..……..
Family history of dementia ……..……..

**Relevant medical history** *(Please also complete table below)*
Subjective memory loss? ……..……..……..
Smoking history (pack years) ……..……..
Use of alcohol (in units per week) ……
Hypertension Yes / No Ischaemic heart disease Yes / No
History of cerebrovascular disease Yes / No Diabetes Yes / No
Rheumatoid arthritis Yes / No Hypercholesterolaemia Yes / No
Infections (last 6 weeks) Yes / No
……
……
……
Trauma (last 6 weeks) Yes / No ……..……
Vaccinations (last 6 weeks) Yes / No ……..
Surgical procedure (last 6 weeks) Yes / No ……...

| Medical conditions | Start | End | Ongoing | Notes |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
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| | | | | |

**Medications**

| Name | Dose | Unit | Freq. | Route | Start | Stop | Indication |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
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**Blood samples**
Time/Date …….. Taken by who ……..

**Testing**

| | | |
|---|---|---|
| Subject: | MOCA ……. | FCSRT-IR ……. |
| Subject/study partner: | CSDD ……. | UPDRS …..……. |
| Study partner: | NPI …….. | CAF …….. |

**Comments** *(please record SAEs here & study forms and report to CI)*
……
……
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……
……

**GP notification letter sent?** ……

**Visit conducted by who** ……. **Signature** ……..…. **Date** ……

# Appendix C

## Immunohistochemistry protocol, Boche laboratory

**1) Rehydration**
Clearene 1: 10 mins
Clearene 2: 10 mins
100% Ethanol 1: 5 mins
100% Ethanol 2: 5 mins
70% Ethanol 2: 5 mins
Tap water rinse then distilled water rinse: 5 mins each
Transfer to green tray and wash with TBS x3

**2) Blocking endogenous peroxidase**: **3% $H_2O_2$** (30% stock) in **Methanol**, 10 mins RT using 500µl (remove 590µl of methanol from 5.9 ml Methanol, then add 590µl $H_2O_2$ (30% stock))

**3) TBS** wash: 3x 10 secs RT

**4) Antigen retrieval** (method depends on the antibody)
- 1 mM **EDTA buffer** pH 8 (0.37 g EDTA in 1L $dH_2O$, pH to 8 with ~ 8 ml of 0.1 M NaOH); microwave with slides (note slow to buffer)
- 10 mM **Citrate buffer** pH 6 (2.1 g citric acid-monoxydrate in 1L $dH_2O$, pH to 6 with ~ 25 ml of 1 M NaOH); mix speed 5
- 0.5% **Pronase**, 20 mins, RT

Microwave method: Slides, in black plastic racks, are placed into the *plastic white square containers* and microwaved (50% power; Sharp microwave, R-27STM-A 800W) in **selected solution** (~ 330 ml) for 25 mins (small slides); after this, the containers are taken out (wear insulated large gloves) and filled with running cold tap water until cooled down; 1-2 mins
Pressure cooker method: see the HRU protocol, 2mins

**5) TBS** wash: 3x 10 secs RT

**6) Blocking solution** saturation step [depends on the host species used for production of secondary antibodies]. 20 mins RT using 500µl **DO NOT WASH**
***Swine anti-rabbit*** – (Dako), HRU blocking medium (freezer, 5ml aliquots)
***Goat anti-mouse*** – (Vector), HRU blocking medium (freezer, 5ml aliquots)
***Rabbit anti-goat*** – (Dako), normal rabbit serum 1:20 in TBS (fridge, 10ml vials)

**7) Primary antibody** (usually kept in LD78, in the fridge or at -20°C)
90 mins RT or O/N fridge or O/N RT

**8) TBS** wash: 3x 10 secs RT

**9) Secondary antibody**
***Swine anti-rabbit*** – Dako 1:400
***Goat anti-mouse*** – Vector 1:800 (HRU) or 1:400 (Boche lab)
***Rabbit anti-goat*** – Dako 1:600
30 mins RT [e.g. if dilution is 1/400 use 1 µl of the **antibody** in 400 µl **TBS** or **Dako diluent**. Prepare ABC 30min before use.

**10) TBS** wash: 3x 10 secs RT

**11) ABC-HRP complex** (A: 1/75 and B: 1/75) Delphine's box, fridge, HRU; mix it well, 30 mins RT (e.g. 5.3 μl of **A** and 5.3 μl **B** in 400 μl of **TBS**

**12) TBS** wash: 3x 10 secs RT

**13)** Chromogenic reaction with **DAB** (Delphine's box, fridge, HRU; according to manufactory's instructions: 2 drops of **buffer**, 4 drops of **DAB**, 2 drops of $H_2O_2$ in 5 ml **distilled water**; duration of the reaction could be 2-15mins, depends on the antibody and tissue). Put slides straight into mounting black slide holder and keep moist in distilled water.

**14)** Stop the DAB reaction with **TBS x3 (wash thoroughly)** and then with **distilled water.**

**15)** Counterstaining with **Haematoxylin** (by the waste sink, beware of sedimentation) 20 secs RT

**16)** Stop the reaction with **tap water** for 5mins

**17) Dehydration** (under the hood on the left; tip the solution out between the steps)
70% Ethanol 2: 1 min
100% Ethanol 1: 1 min
100% Ethanol 2: 1 min
Clearene 1: 3 min
Clearene 2: 3 min
Clearene 3: 3 min

**18)** Mounting with **Pertex** manually (do not put too much of the mount) or using a machine (68mm drops). If using machine do 20 slides at a time and within 10mins clean back of slide with white tissue, reposition coverslip to ensure it covers tissue and lay on blue tissue to dry.

**19)** Check the reaction under the microscope, after at least 1 hour

## Reagents

Fisher Chemical - Citric acid monohydrate, C/6200/53
Fisher Scientific - Ethylenediaminetetraacetic acid (EDTA), FIC-D/0650/50
Histochemical Research Unit blocking medium
Dako - rabbit anti-goat immunoglobulins biotinylated secondary antibody, E0466
Dako - swine anti-rabbit immunoglobulins biotynalated secondary antibody, E0431
Vector - biotynilated goat anti-mouse immunoglobulins secondary antibody, Y0907
Dako - normal rabbit serum, X0902
Sigma - H2O2 solution 30% in H20, H1009
Vectastain® ABC kit, PK6100
Vector - DAB peroxidase substrate kit, SK4100
Dako - antibody dilutent background reducing, S3022

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