University of Southampton

Faculty of Medicine

Clinical and Experimental Sciences

Neuropathology of the Locus Coeruleus in Alzheimer's Disease

by

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Abstract

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The Locus Coeruleus (LC) undergoes extensive neuronal loss in Alzheimer's Disease (AD) early in the disease process. Depletion of cortical noradrenaline (NA) concentration as a result of LC degeneration, has proinflammatory effects on microglia and likely contributes to the neuroinflammation observed in AD. Furthermore, abnormal intracellular tau aggregation associated with AD is first observed within the LC, decades prior to disease onset and LC neuron loss. This suggests neuropathology in AD originates in the LC, however this has been under appreciated and under-investigated because of a lack of non-invasive, direct measures of LC activity.

Using a high-resolution magnetic resonance imaging (MRI) technique, the LC can be visualised *in vivo*. This pilot study assessed the feasibility of detecting signal intensity (SI) changes of the LC using a developed neuromelanin-sensitive imaging protocol in 24 participants with AD (12 mild AD, 12 moderate AD) and 24 age and gender matched cognitively unimpaired subjects. LC-SI was calculated by comparing maxima values in the anatomical location of the LC to a reference region in the adjacent pontine tegmentum to give a contrast ratio (LC-CR). LC-CR was lower in both the mild and moderate AD groups compared to controls (p<0.05), however there was no difference in LC signal between mild and moderate AD groups. This suggests LC signal decreases occur early in the disease and that NM-MRI of the LC could be used as a biomarker for AD diagnosis. LC-CR correlated with SMMSE score (p=0.032) but did not correlate with peripheral inflammatory blood markers or indirect measures of LC activity including pupil size and task-evoked blood pressure changes.

Whilst LC cell loss and p-tau accumulation have been well studied in humans, surprisingly post-mortem studies have not examined inflammatory changes occurring within the LC and how these relate to LC neuron loss, extracellular neuromelanin deposits and changes seen in LC projection areas. Immunohistochemistry was used to examine markers for AD pathology, LC cell integrity, inflammation and neuromelanin in both the LC and temporal cortex of 60 post-mortem tissue samples grouped by disease severity determined by Braak stage (0-II, III-IV and V-VI). In the LC, disease severity was associated with LC neuronal loss (p<0.001), Aβ (p<0.001) and p-tau (p<0.001) accumulation, increased extraneuronal neuromelanin deposits (p<0.001) and increased inflammation evidenced by increased motility of microglia (p<0.001). This correlated with p-tau (p<0.001) and Aβ pathology (p<0.001) but not inflammatory markers in the temporal cortex.

A better understanding of the role of the LC-NA system in AD may inform on whether the pharmacological elevation of NA would be successful in slowing disease progression and at which time-points it should be administered. If NA manipulation was able to delay AD pathology, then early detection and monitoring of LC degeneration *in vivo* using NM-MRI as a biomarker would enable targeting of therapies.

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2. Locus Coeruleus in Alzheimer's Disease Post-mortem study data ARUK

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Research Thesis: Declaration of Authorship

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Signature: ..... Date:.....

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Definitions and Abbreviations

ABC	Avidin-biotin-peroxidase complex
AChEI	Acetylcholinesterase inhibitors
AD	Alzheimer's Disease
ADAS-cog	Alzheimer's Disease Assessment Scale - cognitive subscale
ADCS-ADL	Alzheimer's Disease Co-operative Study - Activities of daily living
aMCI	Amnestic Mild Cognitive Impairment
APOE	Apolipoprotein
APP	Amyloid precursor protein gene
AR	Adrenoreceptor
Aβ	Amyloid-beta
BPM	Beats per minute
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CR	Contrast Ratio
CSF	Cerebrospinal fluid
DA	Dopamine
DAB	3,3’ diaminobenzidine
DβH	Dopamine beta hydroxylase
DDC	Dopamine decarboxylase
DOPA	Dihydroxyphenylalanine
DRN	Dorsal Ralphe Nucleus
EOAD	Early onset Alzheimer's Disease
EWN	Edinger-Westphal Nucleus
FAD	Familial Alzheimer's Disease
FFPE	Formalin-fixed, paraffin embedded
FSE	Fast Spin Echo
GWAS	Genome Wide Association study
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HRA	Health Research Authority
ICF	Informed Consent Form
IHC	Immunohistochemistry
IL	Interleukin
IQR	Interquartile range
LOAD	Late onset Alzheimer's Disease
LC	Locus coeruleus
LLC	Left Locus coeruleus
LPA	Logopenic progressive aphasia
M	Mean
MARC	Memory Assessment & Research Centre
MCI	Mild Cognitive Impairment
MHPG	3-methoxy-4-hydroxyphenylglycol
MoCA	Montreal Cognitive Assessment

MRI	Magnetic Resonance Imaging
NA	Noradrenaline
NET	Norepinephrine Transporter
NFTs	Neurofibrillary tangle
NIA-AA	National Institute on Aging and Alzheimer's Association
NMDA	N-methyl-D-Aspartate
NM-MRI	Neuromelanin-sensitive Magnetic Resonance Imaging
NPI	Neuropsychiatric Inventory
PET	Positron Emission Tomography
PHF	Paired helical filaments
PIS	Participant Information Sheet
PSQI	Pittsburgh Sleep Quality Index
p-tau	Hyperphosphorylated tau
REC	Research Ethics Committee
REM	Rapid Eye Movement
ROI	Region of Interest
RT	Room temperature
SD	Standard Deviation
SHFT	Southern Health Foundation Trust
SI	Signal intensity
SMMSE	Standardised Mini-mental state examination
SN	Substantia Nigra
SWDBB	South West Dementia Brain Bank
SWI	Susceptibility Weighted Imaging
TBS	Tris-buffered saline
TNF-α	Tumour necrosis factor
TSE	Turbo spin echo
UHS	University Hospital Southampton NHS Foundation Trust

Chapter 1 Introduction

1.1 Alzheimer's Disease

1.1.1 Epidemiology and Symptoms

Alzheimer's Disease (AD) is a neurodegenerative disorder affecting approximately 850,000 people in the United Kingdom [1]. Prevalence is expected to increase globally from 55 million in 2021 to 152 million in 2050 (Dementia Factsheet September 2021, World Health Organisation). AD is clinically defined by DSM-IV criteria as a disease with a gradual, continuing decline in a range of multiple cognitive deficits. Whilst the main symptom noted is memory impairment, people can also experience aphasia, apraxia, agnosia and problems with executive function (i.e., planning, organising, sequencing, abstract thinking). These impairments have a significant effect on someone's ability to function socially or occupationally [2]. The gradual decline in cognitive impairment means AD can be considered a spectrum disorder which spans many years, even decades. Greater awareness of AD has led to patients being identified earlier in the disease process. These people are not sufficiently impaired functionally to warrant an AD diagnosis and instead a transitionary diagnosis of 'Mild Cognitive impairment' (MCI) is given. MCI can have multiple aetiologies making it challenging to differentiate those who will go on to develop AD. Diagnosis is made based on clinical history and performance on standardised cognitive tests such as the Mini-Mental State Examination (MMSE). It is estimated that 15-20% of individuals with MCI will convert to AD each year [3,4]. Sub-types of MCI aid prediction of who will develop AD, for example amnestic MCI (aMCI), a MCI group with short-term memory impairment, or prodromal AD, a MCI group with biomarkers for AD (biomarkers for AD are discussed further below in section 1.1.4 Biomarkers).

Almost 20 years ago, acetylcholinesterase inhibitors (AChEIs), i.e., Donepezil, Rivastigmine and Galantamine as well as Memantine (N-methyl-D-Aspartate blockade) were licensed for AD. When tolerated, these can give short-term symptomatic improvements but do not cure or alter the progressive course of dementia. In 2021, Aducanumab, a monoclonal antibody targeting amyloid beta (Aβ) was licenced for use in the USA by the FDA. However its long term effects on disease progression are unclear and further trials will be conducted before it is approved for use in the UK.

1.1.2 Environmental and Genetic Risk Factors

Age is the most important risk factor for the development of AD with rates of the disease increasing exponentially after the age of 65 years and approximately 95% of cases occurring after this age. AD cases that occur after the age of 65 years have been arbitrarily termed 'Late Onset AD (LOAD).' Although the symptomatology is similar, this helps to differentiate them from cases that may have a greater genetic component, occurring under or at the age of 65 years and termed 'Early onset AD (EOAD)'. Lifestyle factors and conditions associated with cardiovascular disease such as type 2 diabetes, obesity, smoking, hypertension and hyperlipidaemia also increase the risk of AD [5].

Bacterial infections such as Chlamydophila Pneumoniae which cause respiratory tract infections such as pneumonia and Spirochetes which causes syphilis are associated with a five-fold and ten-fold increased occurrence of AD respectively, though a causal role of these infections in the progression of AD is uncertain [6]. One historical cohort study of nearly 1 million individuals in the UK over the age of 65 found an increased risk of AD following common infections especially sepsis and pneumonia leading to hospital admission [7]. Additionally chronic periodontitis has been linked with cognitive decline and one of the key pathogens responsible for periodontitis, porphyromonas gingivalis, has been detected in AD brains [5]. Other environmental risk factors include air pollution, metals and pesticides [8].

Twin studies show that AD has a heritability of around 70% [9] and so genetic factors make a substantial contribution to the disease. Genetic factors can be considered deterministic genes i.e., on their own will inevitably cause AD or risk genes that combined with environmental factors increase the risk that an individual will develop AD.

1.1.2.1 Amyloid Precursor Protein (APP)/Presenilin (PSEN) gene mutations

Mutations in three genes, APP, PSEN1 and PSEN2, each resulting in the over production of amyloid-beta (Aβ), have been found to be the cause of most cases of deterministic AD. These autosomal dominant mutations are rare and account for less than 1% of all AD cases. PSEN1 and PSEN2 both produce presenilin which is part of the γ-secretase complex and has a role in cleaving APP into smaller Aβ peptides. Mutations in PSEN1 affect cleaving of APP by γ-secretase resulting in an overproduction of $A\beta_{42}$ and are the most common, accounting for approximately 70% of all autosomal dominant AD cases. Mutations in the APP gene, found on chromosome 21, usually favour cleaving of APP by β or γ-secretases, again resulting in an accumulation of $A\beta_{42}$ [10,11]. Additionally, a high proportion of people with Downs syndrome, who have an extra copy of

chromosome 21, have more amyloid plaque deposits and many go on to develop AD in their midlife. The discovery of these gene mutations led to the proposed 'amyloid cascade hypothesis.'

1.1.2.2 APOE ε4

A genome wide association study (GWAS) found Apolipoprotein (APOE) to be the major risk gene for developing AD [12]. APOE is a protein which combines with lipids to form lipoproteins which package cholesterol and carry it around the bloodstream. APOE has 3 major alleles which encode 3 different isoforms of apolipoprotein: ε2, ε3 and ε4 with 6 possible phenotypes. In Caucasian populations, the ε3 allele is the most common (75%), followed by ε4 (15%) then ε2 (8%) [13]. Those who are carriers of an ε4 allele have an increased genetic risk for developing AD which is also associated with an earlier onset of symptoms [13,14]. Being homozygous for ε4 (approximately 2% of the population) confers the greatest risk, though not everyone with 2 copies of the ε4 allele will develop AD [15]. Having a copy of the ε2 allele is thought to offer some protection from the disease or at least delay symptoms until after 90 years old [16]. Though the mechanism between APOE and Aβ deposition is not understood [17], APOE has been found to co-localise with Aβ in plaques and in cerebral blood vessels [18]. The APOE ε4 allele is associated with increased Aβ plaque deposition [19] and increased number of neurofibrillary tangles [20].

1.1.2.3 Genes related to inflammation/innate immunity

In addition to rare genetic mutations that give rise to rare deterministic cases of AD (e.g., PSEN1 and APP) and common genetic risk factors that give rise to common forms of AD (APOE ε4) more recent genetic research has identified large numbers of rarer mutations that individually contribute to a small increased risk of developing AD, which could be substantially increased when combined. Interestingly these mutations discovered in GWAS studies have identified polymorphisms in genes that are related to innate immunity, microglial activation and complement activation and are associated with a small increased risk of developing late onset AD[21-25]. Of these mutations in the CD33 gene and TREM2 gene have received the most attention[26].

1.1.3 Pathology

1.1.3.1 Neuronal and Synaptic Degeneration

Cognitive decline in AD is associated with progressive neuronal and synaptic loss evident on both Magnetic Resonance Imaging (MRI) scans and at post-mortem as global cerebral atrophy, which is most prominent in the hippocampus and temporal lobe [27]. Additionally, there is an abnormal deposition of extracellular beta amyloid (Aβ) peptide as plaques and intracellular

hyperphosphorylated tau fibrils which accumulate in the neurons; neurofibrillary tangles (NFTs) in the cell body, neuropil threads in the dendrites and dystrophic neurites in neuronal processes around Aβ plaques to form neuritic plaques [28]. Bilateral brain areas are often affected symmetrically [14].

1.1.3.2 Amyloid-beta (Aβ)

The amyloid precursor protein (APP) gene, located on Chromosome 21 is expressed by many cell types, although the function of APP remains unknown. 40 to 42 length amino acid, soluble Aβ peptides are proteolytically cleaved by β-secretase and γ-secretase from APP to form insoluble fibrils of β-pleated sheets. These fibrils aggregate into microscopic senile plaques which have a dense core of fibrils which stream out in a star shape [29]. In AD these plaques initially appear in the hippocampus, amygdala and temporal cortex, then later accumulate in the frontal and parietal cortices before finally affecting brainstem nuclei.

As discussed earlier (1.1.2.1) in FAD, autosomal dominant mutations in the APP gene cause an overproduction of $A\beta_{42}$ which results in significant plaque deposits, also seen in sporadic AD. Identification of APP mutations and other autosomal dominant gene mutations, led to the 'amyloid cascade hypothesis' which has dominated research for the past 20 years and theorises that Aβ is the initial event causing abnormal tau pathology and neuronal loss [30,31]. However, in sporadic AD, over cleaving of APP due to gene mutations is not the cause of Aβ accumulation.

Transgenic mice with human APP gene mutations also accumulate Aβ plaques with age and show impairments in working memory tasks such as the Morris Water Maze and so have been used as murine AD models to study the disease and test potential treatments [32]. Aβ plaques in transgenic mice appear as early as 6 months and emerge with a dense core of fibrils. Neuritic plaques can form within one day and reach full size within 24 hours [33], but have also been observed to form over several weeks before reaching full-size [34]. Daily imaging of new plaques using multiphoton microscopy revealed that previously normal neurites twist round the plaque as it develops, rapidly becoming dystrophic. This supports the cascade hypothesis as it implies that Aβ plaques occur prior to neuronal degeneration [33]. However in these models the mice do not accumulate NFTs or experience the neuronal loss observed in humans, questioning the role Aβ plays in the development of tau pathology and neurodegeneration [35]. In humans, dystrophic neurons are often observed close to dense core plaques [36] suggesting that the plaques cause synaptic loss. Additionally, the plaques evoke an inflammatory glial response (discussed in section 1.1.5.3) both of which result in neurodegeneration. Diffuse plaques also observed in the neocortex are not surrounded by dystrophic neurons [36], do not evoke the same glial response or result in synaptic

loss [37] and are less likely to be associated with poor cognition [38]. In AD mice, diffuse plaques account for less than 10% of total amyloid stained [29].

In sporadic AD, it is not known whether it is overproduction of Aβ or an inability to remove it that results in the abnormal accumulation of plaques. Vascular damage which is often co-morbidly observed with AD, may promote the upregulation of APP resulting in subsequent aggregates of Aβ[17]. Disease modifying trials targeting amyloid pathology have so far been unsuccessful in stopping or slowing further cognitive impairment despite their success in significantly reducing the number of Aβ plaques [39]. Some argue that treatment is occurring too late in the disease process and that rather than the fibrillary plaques, it is the soluble oligomers of Aβ that are toxic to the cell. Indeed the soluble oligomers are able to diffuse through the brain parenchyma and across synapses [40] and the number of oligomers correlates better with cognitive decline than the number of plaques [41]. Furthermore, injecting rats with Aβ oligomers without senile plaques inhibits long term potentiation in the hippocampus [42].

However both neuritic and diffuse plaques also accumulate as part of normal ageing with one study finding 57% of a sample of elderly cognitively unimpaired brains to contain them [38]. Furthermore in another study, one-third of cognitively normal participants had moderately severe Aβ plaque scores [43], therefore questioning both the role of plaques in developing dementia and the usefulness of amyloid as a biomarker.

1.1.3.3 Tau

Tau is a cytoskeletal, microtubule-associated protein (MAPT) and its main function is to stabilise axonal microtubules. There are six isoforms of tau with chain lengths of between 352 and 441 amino acids. They include repeating units which are able to bind to the negatively charged microtubule. Three of the isoforms contain three repeating units (known as 3Repeat tau) and three of the isoforms contain four repeating units (known as 4Repeat tau). 4Repeat tau binds more readily to the microtubules because of its extra binding domain. Tau has 79 potential serine and threonine phosphorylation sites on the longest tau isoform. In homeostasis, tau phosphorylation is maintained by tau-related kinases and phosphatases. However, in AD, the sites become fully saturated, and tau is hyperphosphorylated (p-tau) and is no longer able to bind to microtubules and maintain their stability. This unbound p-tau polymerizes to form paired helical filaments (PHF) which aggregate as insoluble neurofibrillary tangles (NFTs) over time. It is not known why tau becomes hyperphosphorylated but presumed that a combination of genetic and environmental factors causes the dysregulation of tau-related kinases and phosphatases.

Histochemistry reveals that NFTs follow a progressive, predictable pattern throughout the disease process with regards to their severity and location in the cortex. This has led to the development of a classification system which outlines different levels of pathology at different stages, known as Braak stages I-VI, (see Figure 1). At Stage I, NFTs are observed in the transentorhinal cortex (parahippocampal gyrus); at Stage II, NFTs are found in the entorhinal cortex including parts of the thalamus and CA1 of the hippocampus, further hippocampal coverage occurs at Stage III which then additionally affects the basal, frontal and insular cortex by Stages IV and V; by Stage VI, the primary sensory and motor areas are also affected [44]. Cognitive symptoms are not experienced until Stage III or IV where the NFTs affect the hippocampus and therefore tau pathology occurs prior to symptom onset. People with pathology at Braak Stage I-II are considered to be in a preclinical phase of the disease, whereas those at Braak stage III are thought to have prodromal AD [44]. This means there is a window of opportunity in which to treat the disease before cognition is affected and therefore early detection of AD pathology is key.

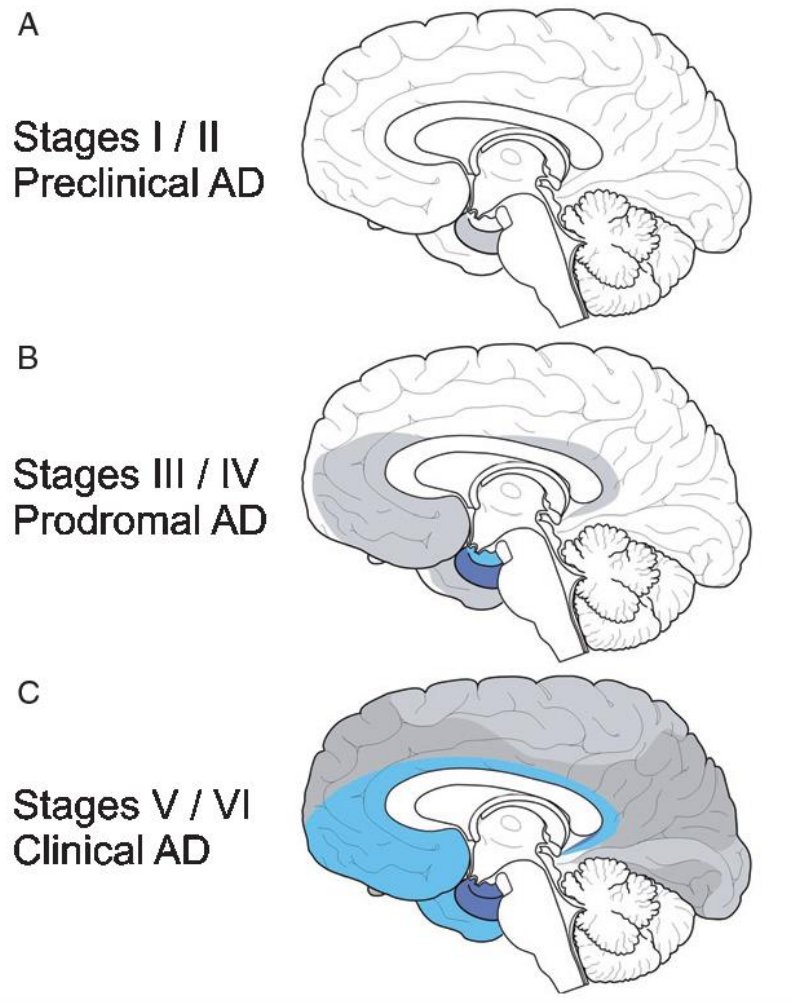


Figure 1. Braak staging classification system to show the topographical spread of tau pathology through AD progression.
Image obtained from Rueb et al. [45].

As abnormal amyloid and tau accumulations begin in anatomically different places, many have tried to establish the relationship between these pathologies, which is today still not fully understood [17]. Tau pathology in the brainstem and medial temporal lobe tends to precede Aβ plaque deposits [14] and tau pathology correlates better with cognitive decline in dementia than Aβ plaque deposition [46,47], both questioning the validity of the amyloid cascade hypothesis. However, others maintain that whilst neurodegeneration related to tau pathology may be causing cognitive impairment, amyloid is the critical factor which precipitates the spread of tau. In vitro experiments certainly suggest that when added to cell cultures, Aβ is capable of causing tau hyperphosphorylation in human hippocampal and cortical neurons [48,49]. However often this is in the form of synthetic Aβ and is added at varying concentrations [50]. Evidence from human biomarker studies discussed further below also suggests that amyloid precedes tau deposition, supporting the amyloid cascade hypothesis.

1.1.4 Biomarkers

Current evidence suggests that the neuropathology associated with AD is present at a preclinical stage possibly years before the manifestation of any symptoms and so early detection is key to any potential treatment. Building on previous guidelines from the National Institute on Aging and Alzheimer's Association (NIA-AAA), a diagnostic framework for use in a research setting was released in 2018 and was based on biomarkers. The framework suggests assessing whether the person is positive or negative for biomarkers of amyloid, tau or neurodegeneration and giving them an 'ATN' profile. The profile is combined with the level of cognitive impairment to provide a more accurate diagnosis, with the emphasis being on amyloid biomarkers which must be positive to be classified as AD [51].

Biomarkers of Aβ build up in the brain include reductions in $A\beta_{42}$ measured in the cerebrospinal fluid (CSF) by up to 50% compared to controls [52] and increased amyloid binding measured using amyloid brain Positron Emission Tomography (PET) scans. For PET scans Pittsburgh Compound B (PiB) which binds to Aβ is the most commonly used ligand and correlates well with post-mortem amyloid deposition [53]. In AD PiB binding increases with disease severity [54] and MCI patients with high PiB binding are more likely to convert to AD [55]. CSF and PET biomarkers of amyloid are highly correlated with each other within the same individuals [56] and are typically used to determine who to include in clinical trials for disease modifying treatments that target amyloid when symptoms are mild, and it is unclear whether the cognitive impairment is due to AD. However, CSF sampling is an invasive procedure and PET scanning involves exposure to radiation which can be problematic if repeated scans are required.

High levels of total tau (t-tau) and phosphorylated tau (p-tau) in the CSF are associated with AD, and correlate with hippocampal atrophy [57] and NFT burden at post-mortem. However although these markers have high sensitivity, they have low specificity, also being indicative of other tauopathies, so can be used to rule out AD rather than diagnose it [58]. Abnormalities in CSF $A\beta_{42}$ precede abnormalities in CSF tau [59]. More recently tau PET ligands have been developed, and initial studies looking at both tau and amyloid using PET imaging, also suggest that it is unusual to see abnormal tau without abnormal amyloid whilst conversely amyloid is observed without tau [60] supporting the theory that amyloid accumulation is an initiating factor in AD. It is possible that a threshold for Aβ needs to be reached before tau pathology spreads outside of the medial temporal lobe [61]. Interestingly amyloid deposition is observed in some people with normal cognition. Some claim these people have prodromal AD and are being detected before they display symptoms, whereas others feel this is further evidence of tau pathology being better correlated with poorer cognition.

Hippocampal and cerebral atrophy is only seen on MRI and CT scans once cognitive impairment is already apparent and currently these scans are only used to exclude other causes or monitor progression. MRI atrophy correlates with NFT accumulation rather than Aβ load [59,62,63]. On Fluorodeoxyglucose (FDG)-PET AD patients show decreased glucose uptake in the posterior cingulate cortex and the temporoparietal cortex indicating a lower rate of cerebral metabolism in these areas, thought to reflect synapse loss [64].

One area more recently being investigated is measuring microglial activation in AD, detected by PET using TSPO ligands [65] and may aid our understanding of the immune system's role in AD.

The combined data from these biomarkers can be used to predict who with MCI is more likely to develop AD [15,30]. However, the current markers are invasive and still do not allow for detection early enough in the disease process. Improved early-stage biomarkers are needed both to allow the patient to access support to live with their condition and to have the best chance of success with any potential new treatments [66].

1.1.5 Neuroinflammation

Neuroinflammation refers to the activation of immune cells in the central nervous system (CNS) as a result of injury, pathogen or tissue damage. Changes in immune cell morphology and signalling is a hallmark of AD and previously thought to be in response to neuropathology associated with AD. However the role the immune system plays in the development and progression of AD has been underestimated and although not fully understood is now being investigated further [67].

1.1.5.1 Microglia

Microglia which make up 10-15% of all cells in the brain are the resident macrophages of the CNS. Microglia numbers are thought to be stable over time due to a balance of self-renewal and apoptosis [68] and they are the only cells identified to regenerate in the CNS [69]. Physiological microglia have a ramified morphology consisting of a small rod-shaped cell body which stays in place and multiple dendrites with many branches which extend in all directions to survey the surrounding area for pathogens and damaged neurons [70]. Time lapse experiments observing microglia in mice show that the somata hardly moves. However, although there are a few branches that remain relatively fixed in space, most of the microglial processes are highly motile, continuously extending and retracting over a period of minutes. If one of the processes meets a process of another microglia, they repel each other so they can maintain their own territories (although these territories are constantly evolving). By this mechanism, it has been estimated that the entire brain can be surveyed every few hours [71]. Through their many receptors, microglia are very sensitive to changes in their environment and can adopt a range of phenotypes depending on the signals they receive. When pro-inflammatory cytokines, pathogens or damaged tissue are detected, microglia become reactive. In this active phenotype, microglia exhibit amoeboid pathology with an enlarged cell body and shorter dendrites with less branches. Nearby microglia locate to the site of injury whereas microglia in other brain areas are unaffected. At the site of injury they proliferate and phagocytose cells, engulfing tissue and transporting it to the cell body [71]. Microglia make contact with other cells including astrocytes and cells of the blood brain barrier [71] as well as brief, frequent contact with neuronal synapses. When damage to a synapse is detected longer contact is made and the synapse in the damaged cell subsequently disappears, suggesting microglia are responsible for their removal [72]. There are many states of activation and microglia are able to switch between multiple phenotypes in response to signals they receive and during different stages of an inflammatory response [73].

1.1.5.2 Cytokines and Chemokines

Microglia (as well as astrocytes and neurons) also secrete cytokines, which are chemical messengers that are integral to the immune system enabling communication between different immune cells when pathogens are detected. Different cytokines have predominantly proinflammatory or anti-inflammatory functions. Activated microglia have different phenotypes depending on the stimulus they encounter in their environment. Although now thought to be oversimplified, a classification system divides these into two phenotypic groups or activation states. M1 or classical activation, results in expression of proinflammatory cytokines such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) and is associated

with tissue damage. Whereas M2 or alternative activation in which anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 are secreted, is thought to be associated with tissue repair [74].

Stimulation of cells by a pathogen or by cytokines also causes secretion of chemokines, which are chemoattractant cytokines that predominantly regulate the migration of immune cells via chemotaxis. Many cells including microglia and neurons have chemokine receptors which are a family of g-coupled receptors. Different chemokines are able to bind to different receptors, but each chemokine can usually bind to many receptors. This means the response can be specific as the many combinations enable precise and complex signalling. Some chemokines are pro-inflammatory, recruiting immune cells to a site of infection, whilst others are homeostatic.

1.1.5.3 Microglia in AD

Microglial activation measured by Iba1 expression is increased in AD mice compared to wild-type mice and co-localised with staining for Aβ42 [75]. In AD mice models, microglia are attracted to Aβ plaques; between 3 and 20 microglia surround each Aβ plaque, covering and penetrating it with their processes [29]. Many of these microglia have an amoeboid phenotype with observable inclusions suggesting they are phagocytic (although phagocytic microglia were most frequently observed adjacent to dystrophic neurons) [29]. Interestingly diffuse plaques observed with ageing are not associated with this microglia response [29]. Similarly a 3D reconstruction of microglial cells in humans showed 5 to 6 microglial cells around each amyloid plaque with the authors describing the plaque as an amyloid star with the microglial processes attached to the points [76]. In one human post-mortem study of 50 cases including both unimpaired and AD brains, 90% of neuritic plaques were associated with microglia [77]. Microglia rated visually as being morphologically activated due to their ramified shape are associated with increased levels of Aβ and NFTs, the strongest correlation being with Aβ in the cortex but not in subcortical structures examined [78].

Neuroinflammation is observable by PET imaging in humans *in vivo* with the use of TSPO radioligands (e.g., PK11195) which bind to reactive microglia. Increased uptake of 20-35% of the TSPO tracer was seen in areas which show increased Aβ deposition on amyloid PET scans and uptake also correlated with lower MMSE scores [79]. One longitudinal study comparing baseline PET scans measuring amyloid, glucose metabolism and microglial activation with scans repeated 16 months later in AD and control participants, found not only did the AD group have higher baseline levels of microglial activation, these levels were also increased further at the second scan[80]. Whilst these findings have been replicated [81], other studies comparing TSPO uptake have failed to find differences in people with AD, MCI or controls [82]. This may be because of the use of different methodologies e.g. in choosing which reference regions to sample and unfortunately there are challenges in accurately quantifying neuroinflammation using this technique including

poor signal to noise ratio, individual differences in affinity for TSPO binding and inability for the ligand to distinguish between different microglial phenotypes [83]. Non TSPO tracers e.g. cannabinoid type 2 receptor (CB2R) which is expressed by microglia and typically upregulated in AD are also being developed [84].

Findings from studies comparing TSPO uptake with tracers that indicate tau pathology are also mixed. Although some show microglial activation to correlate with tau pathology [85], one study using PET to compare levels of tau, amyloid and inflammation in MCI and AD participants found only amyloid was correlated with inflammation [86]. Interestingly high levels of inflammation were seen with those with high amyloid but low tau signal at the MCI stage with the researchers concluding that inflammation is an early event that precedes tau deposition [86]. This fits with the hypothesis that neuroinflammation may be the mediator between amyloid and tau pathology, however more longitudinal studies are needed to confirm this [83].

In an experimental model of cultured rat microglial cells, the addition of a senile plaque core (isolated from the frontal and temporal cortices of AD patients), activated microglia towards a phagocytic phenotype to clear the plaque [87]. However in *in vivo* models, despite microglia being rapidly attracted to the plaque within 24 hours they do not phagocytose Aβ [33]. The size of the plaques do not increase however, which suggests microglia have a role in controlling their growth, creating a barrier around them [88]. This is supported by an *in vivo* time lapse experiment in transgenic mice which showed microglia clustering around plaques remain highly motile and show signs of Aβ phagocytosis (evidenced as inclusions in their processes). Therefore microglia could be controlling the development of the plaque, despite being unable to clear it away [89].

However, this has remained controversial, and an alternative position is that microglial phagocytic ability is impaired in AD. When microglia bind to Aβ via cell-surface receptors they secrete proinflammatory cytokines [90] and in AD mice models there is increased expression of proinflammatory cytokines e.g. IL-1β and TNF-α [91] which correlate with Aβ levels [75,92]. Similarly, human microglia cultures obtained from AD and control brains and exposed to Aβ, show dose dependent increases in IL-6, IL-1β and TNF-α [93]. In AD mice, microglia incubated with TNF-α show reduced phagocytic ability [91] and the phagocytic ability of microglia in the vicinity of Aβ plaques is impaired in AD mice [94]. This suggests microglial dysfunction may be responsible for the lack of Aβ clearance [94] and a feedback loop has been proposed in which Aβ causes secretion of cytokines which in turn inhibit Aβ clearance and therefore promote Aβ accumulation [91]. This is supported from findings from GWAS studies which suggest that genes that result in microglial dysfunction are a risk factor for developing AD [95]. Furthermore, one study found microglia co-localised with Aβ to be ramified and found that microglia located near tau inclusions were more likely to be

dystrophic than activated, and this dystrophy appears to precede tau pathology (when using Braak staging). They suggest that a loss of microglial protective function linked to aging is responsible for AD progression [96].

Inflammation in response to a pathogen or tissue damage usually resolves once the offender is removed, however it is thought that in AD, microglia are chronically activated or 'primed' by Aβ and damaged neurons [67]. This results in the over-secretion of inflammatory cytokines, leading to recruitment of more immune cells and contributes to synapse and neuron loss, as well as exacerbating tau pathology [97]. MCI and AD patients have elevated levels of both proinflammatory and anti-inflammatory cytokines in their CSF [98] suggesting an imbalance in the immune system, but it is unclear as to whether this is cause or effect of the disease [99]. However, when measuring cytokine levels at baseline and at 12 months, increased proinflammatory cytokine expression was associated with less cognitive decline suggesting a protective role of the immune system and so there is still some debate about how harmful this proinflammatory environment is [98]. The variation in results may be due to the timepoint in disease when sampled and so more longitudinal studies are needed for this to be a reliable method of measuring inflammation in vivo[67].

Systemic infections are known to worsen cognitive decline and one explanation is that increases in peripheral proinflammatory cytokines cross the blood-brain barrier where they evoke an exaggerated response from microglia already primed by Aβ accelerating the progression of AD. People with dementia had higher levels of inflammatory proteins such as IL-6, IL-1, TNF-α, in plasma prior to onset [100,101]. One longitudinal study that followed people with AD over 6 months found that those with higher peripheral TNF-α (indicative of systemic inflammation) at baseline had a four-fold increase in rate of cognitive decline and those that also had systemic infections over that time had a ten-fold increase in cognitive decline [102]. Experiments which use LPS to activate microglia in transgenic mice result in increases in IL-1β and TNF-α secretion as well as increases in cognitive decline [103], neuronal death [104] and tau hyperphosphorylation [105]. Some now propose that microglia could be the link between Aβ and tau pathology, with an unhealthy microglial response to Aβ resulting in the spread of tau from the transentorhinal cortex.

Although use of non-steroidal inflammatory drugs (NSAIDs) has been associated with a lower prevalence of AD [106], randomised control trials of anti-inflammatory drug treatment have been unsuccessful in reducing amyloid accumulation or cognitive decline [107]. It is unclear whether microglia are responding to neurodegeneration or are causal in neuron loss. It is possible that a combination of an individual's susceptibility to microglial dysfunction with age and an ineffective microglial response to Aβ, as well as Aβ and tau deposition are necessary for AD to occur [95].

1.2 The Locus Coeruleus

1.2.1 Anatomy

The locus coeruleus (LC) is a rod-shaped cluster of noradrenergic cells located bilaterally in the brainstem. In the healthy brain, it is between 12 and 17mm long and approximately 2.5mm wide[108,109]. The LC cells start at the level of the inferior colliculi and extend down the dorsal pons, alongside the lateral floor of the fourth ventricle which displaces the cells laterally in a horn shape[108,110], see Figure 2. As they extend caudally the cells become more compact, being most concentrated in the lower middle aspect of the LC which incidentally appears thinner as the neurons form a more cylindrical shape [109]. The estimated number of LC cells on each side varies between studies and participants but has generally been estimated to be between 11,000 and 25,000 with no asymmetries between the left LC (LLC) and right LC (RLC) [108,110-113] and no gender differences [113]. Whilst differences in methodology may be able to account for some of the variation between studies, there also appears to be significant individual variation in LC cell number within studies [112] with one study showing individual cell counts in healthy individuals to range between 7000 and 62,000 cells on each side [114]. It is also unclear from previous studies whether there is a greater concentration of LC cells caudally [108,115], or in the middle of the LC [110].

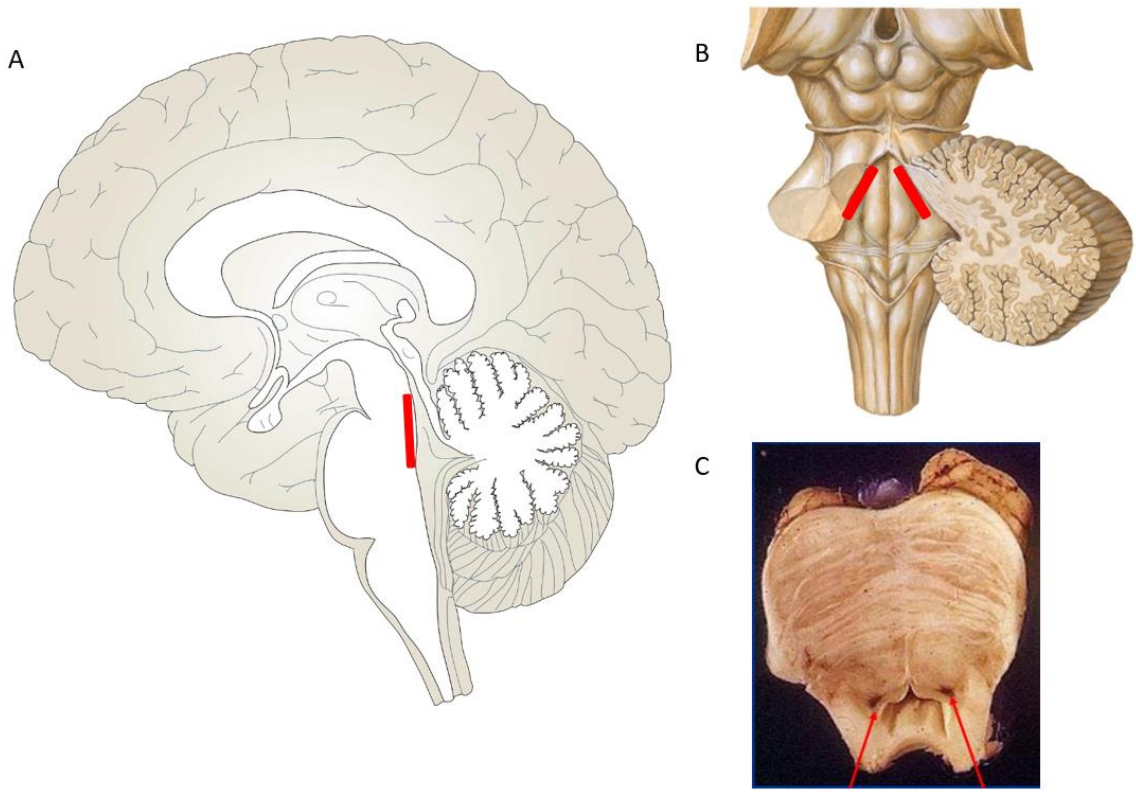


Figure 2. Sagittal (A), coronal posterior (B) and axial (C) views of the brainstem to show the location of the locus coeruleus (red).

Some studies indicate that the number of LC cells decreases in old age [112,116-119], however the decrease in cell number often only tends to be significant when comparing samples from very old brains, e.g. those aged over 90 who often have significantly lower brain weights in general, to those under the age of 60 [108,112,116]. When observed, cell loss in older adults is more prominent in the rostral area of the LC [116,120,121] and is not mirrored in other brainstem nuclei except the substantia nigra (SN). Another study additionally noted that the size of the cell soma was significantly smaller in older adult samples [108], however it employed a very small sample size (*n*=6). Other studies which boast the use of unbiased stereological counting methods or that have ensured that cases used were pathologically normal at death have not observed an age-related effect [109,111,114,122,123]. The large individual variation in cell counts also reported between studies, makes conclusions about neuronal loss in ageing difficult except in the very old.

Most cells of the LC are large multipolar, pigmented cells with round or oval somas with a diameter between 35µm and 45µm, though fusiform cells between 15µm and 25µm wide have also been observed. All LC cells have multiple, long dendrites and axons which join nearby noradrenergic fibres [27,110,124].

The LC is part of the isodendritic network which is made up of four brainstem nuclei that each use and maintain four of the neurotransmitter systems widely used in the brain. These are the LC, which synthesises Noradrenaline (NA); the SN, which synthesises dopamine (DA); the basal nucleus of Meynert, which is cholinergic; and the dorsal raphe nucleus (DRN), which produces serotonin [27].

The LC cell axons project to almost every brain region and to the spinal cord. Autoradiography used to study these projections in the rat [125,126] revealed two main bundles, the ventral bundle which mainly projects to the spinal cord and the dorsal bundle which projects to the cortex and the hippocampus [127]. The anterior part of the LC contains a cluster of cells that project to the hypothalamus [128]. Similar projections from the LC have been observed in primates [129] and suggests that the projections are spatially organised at least to some extent.

The long reaching unmyelinated projections of the LC can communicate both monosynaptically (i.e., neuron to neuron) or by volume transmission, in which neurotransmitters are also released from varicosities along the axon. Many of these varicosities do not make contact with other synapses so NA diffuses into the surrounding space where it is able to act on large volumes of neurons, glial cells and circulating blood [130]. This means that despite its small size the LC can project to most parts of the cerebral cortex and spinal cord innervating the brain with NA. Additionally, the LC can mediate the speed with which it releases NA with two distinct modes of firing, phasic transmission and tonic transmission. Whilst phasic transmission involves the release

of a large amount of NA by a short burst of firing, tonic transmission describes the steady production and release of NA from the LC. Therefore, despite its size, the complexity of the LC enables it to regulate a number of functions.

1.2.2 Neuromelanin

A significant characteristic of the LC is that the cells contain a high concentration of neuromelanin (NM), a dark brown, granular pigment. Although NM is found in many neurons, it is only in the LC and the SN that it is found at such high concentrations that it can be visualised macroscopically Concentrated NM accumulating from the production of NA has a blue hue giving the locus coeruleus its name, being Latin for 'blue spot.' Likewise, the concentrated area of NM resulting from dopamine synthesis has a black colour, with "substantia nigra" being Latin for 'black substance.' NM is present in almost all LC cells and thus is an accurate marker for counting LC cells, comparable to using dopamine-beta hydroxylase (DβH) to count noradrenergic LC cells[110,131,132]. Only small amounts are seen in the SN and LC in early years [133], though NM accumulates at an earlier age in the LC than in the SN. The concentration then increases with age until approximately age 60 then starts to decline [116,134,135].

Whilst most research has focused on the analysis of NM in the SN, most likely because of methodological difficulties in isolating NM from the LC due to its size and location; NM in both locations is thought to be structurally similar. Granules are approximately 30μm in size, are insoluble and located within double-membrane organelles in the cytoplasm of the cell body, typically occupying at least half of the cytoplasm [110], and is absent from dendrites [136]. Electron microscopy studies show NM has a pheomelanin core, eumelanin surface and lipid component [137,138] and is thought to have similar properties to peripheral melanins in the skin [139].

Whilst it is known that melanin pigments in the skin are produced in melanocytes via the enzyme tyrosinase, the mechanisms for NM synthesis are not fully understood, as tyrosinase is rarely found in the brain [140]. It is generally accepted that NM is synthesised by the oxidative polymerisation of excess dopamine and NA by iron catalysts and is therefore a by-product of monoamine synthesis [141]. NM accumulates within autophagic organelles that fuse with lysosomes and autophagic vacuoles that have lots of lipid bodies, see Figure 3 [136]. This removal of metals and excess DA/NA from the cytosol into membrane bound organelles is thought to be neuroprotective, preventing them from causing cellular damage via oxidative stress [142,143]. However it is unclear why NM only appears in some dopaminergic/noradrenergic neurons, suggesting production is in some way regulated by yet unknown enzymes as it is for peripheral melanins, in addition to iron [139,144]. The catecholaminergic neurons of rodents do not contain NM

and although it can be seen in many other primates, the amount is greatly reduced compared to humans [139,145-147], again suggesting that monoamine synthesis can occur without resulting in NM granule accumulation [139]. Additionally, NM in humans is not observed during the first few years of life, taking a while to accumulate and so it may be that rodents and other animals simply do not live long enough to accumulate NM. This makes it difficult to study the function of NM in common laboratory animals.

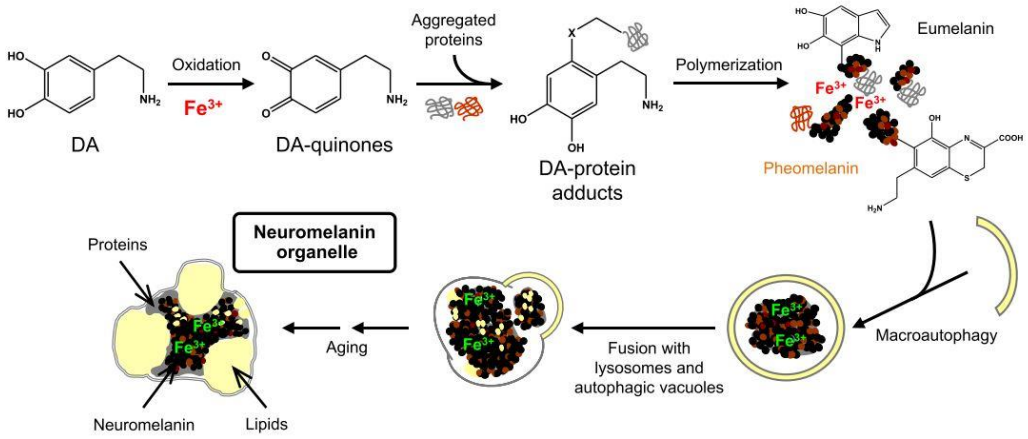


Figure 3. Illustration of neuromelanin synthesis.

Image obtained from Sulzer et al. 2018 [136].

1.2.3 Role of LC-NA system

1.2.3.1 Noradrenaline

The LC is the principal site where the neurotransmitter NA is synthesised in the brain, and the only source of cortical NA as NA is unable to cross the blood-brain-barrier [148]. As shown in Figure 4, NA is formed from L-tyrosine which is either found in the diet or arises from the conversion from phenylalanine by phenylalanine hydroxylase. Tyrosine hydroxylase converts tyrosine to DOPA (dihydroxyphenylalanine) which is in turn converted to dopamine by Dopamine decarboxylase (DDC) before being contained in storage vesicles. Some vesicles contain DβH and convert the dopamine to Noradrenaline. DβH can therefore be used as a marker of noradrenergic cells. The LC cells release NA from the vesicles into the synaptic cleft via exocytosis. There it is able to bind to G-coupled α1-, α2- or β-adrenoreceptors (AR) found both on the LC cells and on microglial and neuronal cells in the many brain areas NA is projected to throughout the cortex [149]. Typically activating α2-ARs with NA has an inhibitory effect on the cell of the receptor, whereas activating α1- or β-ARs with NA usually has an excitatory effect on the cell. Many brain regions express both types of receptor meaning they can be both inhibited or excited by LC activity [150]. LC cells themselves have both of these receptors and binding of NA to α2-ARs on LC cells inhibits their

release of NA whereas binding of β2-adrenoreceptors on LC cells increases their TH expression and therefore increases release of NA. Therefore α2-AR antagonists (e.g., mirtazapine, yohimbine and phentolamine) increase release of NA as the LC cells do not get the signal to stop releasing it whereas agonists such as Clonidine inhibit the release of NA from the LC.

NA can either be 1) degraded by monoamine oxidase which converts NA into the cytoplasm of DHPG (3,3-dihydrixyphenylglycol) and then into MHPG (3-methoxy-4-hydroxyphenylglycol) by COMT (catechol-O-methyl transferase) or 2) taken back into the presynaptic LC neuron via a NA transporter (NET). MAO inhibitors, such as pargyline and NA uptake inhibitors, for example desipramine and reboxetine, both increase extracellular NA. L-DOPS (L-threo-dihydroxyphenylserine) is a synthetic amino acid precursor can also increase NA concentration in the brain. It can be administered orally as it is able to permeate the blood-brain barrier where it is broken down by DDC into NA.

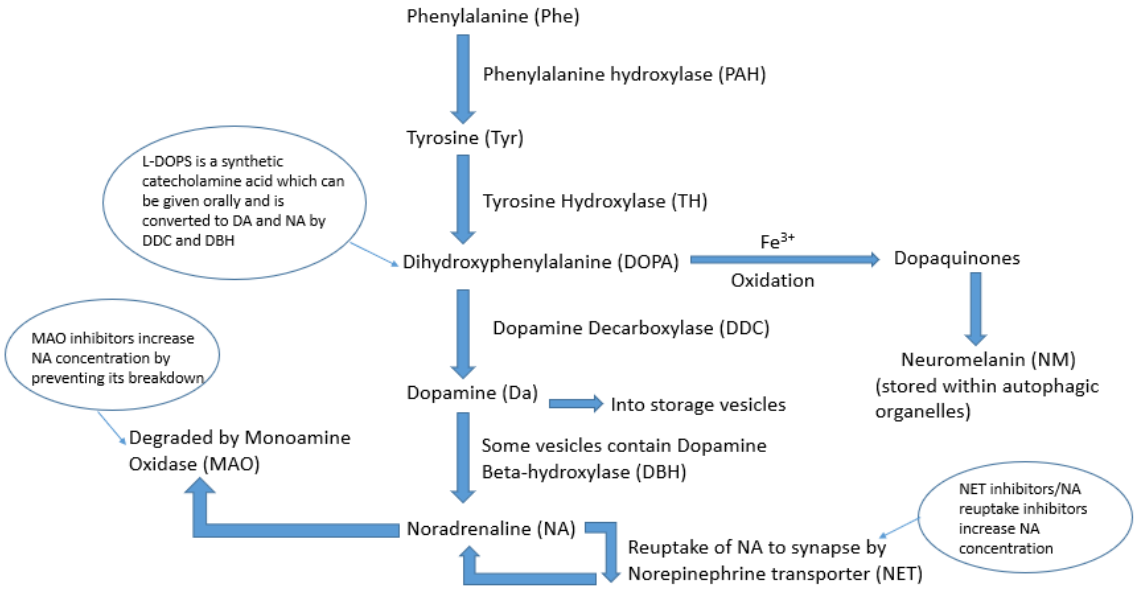


Figure 4. Schematic of noradrenaline synthesis in the Locus Coeruleus.

Phenylalanine is converted to tyrosine (Tyr) by phenylalanine hydroxylase (PAH). This is then converted to Dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH) which is in turn converted to Dopamine (DA) by the enzyme Dopamine Decarboxylase (DDC). Dopamine is taken up into storage vesicles, some of which contain Dopamine Beta-hydroxylase (DBH) which converts dopamine to noradrenaline (NA). Noradrenaline is either taken back up to the synapse by Norepinephrine Transporter (NET) or degraded by Monoamine Oxidase (MAO). Excess cytosolic dopamine and noradrenaline is oxidised by iron catalysts into dopaquinones which are stored as Neuromelanin contained within autophagic organelles [151].

1.2.3.2 NA, Autonomic Function and Arousal

Noradrenaline is the main neurotransmitter that modulates sympathetic nervous system activity preparing the body for 'fight or flight' in response to danger. When a threat is detected, the LC activates α1-adrenoreceptors in the amygdala and the paraventricular nucleus of the hypothalamus via its noradrenergic projections. This further increases sympathetic nervous system activity by activating the adrenal-cortical system signalling the adrenal medulla to release NA and adrenaline, as well as other hormones into the bloodstream [152]. NA release triggers vasoconstriction diverting blood away from the gastro-intestinal tract and skin towards the skeletal muscles and lungs, increasing blood pressure as well as increasing the rate and contractibility force of the heart. These cardiovascular changes can also be achieved by electrical stimulation of electrodes in the LC [153,154]. NA also causes the pupils to dilate to allow more light in, enhancing vision for long distances. The role of NA can be demonstrated by pharmacological manipulation by Clonidine, an α_2-adrenoreceptor agonist which directly inhibits noradrenergic activity in the LC [155,156]. Clonidine is an anti-hypertensive medication, lowering blood pressure and heart rate as well as decreasing pupil diameter, having a sedatory effect in humans [157-159]. Furthermore, Yohimbine, which increases noradrenergic activity causes anxiety in humans probably due to over stimulation of the amygdala from the LC [160].

Through the release of NA the LC also plays a major part in the regulation of arousal [152]. The LC has a well-researched role as a wakefulness promoting nucleus, having an excitatory effect on other wakefulness promoting nuclei through its supply of NA. Additionally when the LC is active, NA inhibits the GABAergic neurones of the ventrolateral preoptic area of the hypothalamus (VLPO) that it projects to, an area highly active in Rapid Eye Movement (REM) sleep [161,162]. The LC is virtually silent in REM sleep and quiet during sleep-wakefulness-state [163] and is thought to have a role in causing the atonia observed during normal REM sleep [164]. Single cell LC recordings in monkeys found pauses in LC activity to be associated with drowsiness [165]. Furthermore, electrical stimulation of the LC in one man resulted in significant sleep disruption and decreased periods of REM sleep [166].

However not only is the LC key to the circadian regulation of wakefulness, its' influences are thought to extend further than this, actually maintaining alertness and arousal levels [167]. Single cell recordings from monkeys show there is an increase in LC activity when completing tasks requiring vigilance [165] or when responding to complex novel stimuli [163]. When cats are conditioned to a stimulus with a negative event, e.g., an air puff, there is an increase in LC activity. However this increase in LC activity does not occur when the stimulus is associated with reward[168].

It is thought that the rostral and middle areas of the LC regulate attention and circadian rhythms through projections to the cortex and hippocampus whereas the caudal part of the LC regulates respiratory, cardiovascular and GI activities.

1.2.3.3 The pupil and the LC-NA system

Pupil size is dependent on autonomic innervation with parasympathetic activation of the sphincter muscle causing pupil constriction and adrenergic sympathetic innervation of the iris dilator muscle causing mydriasis [169]. Pupil constriction in response to light is mainly due to parasympathetic influence on the Edinger-Westphal Nucleus (EWN), see Figure 5. However LC activation can inhibit this response by stimulating α2-ARs on the EWN, which has an inhibitory effect, therefore inhibiting pupil constriction [152]. Unilateral stimulation of the LC in rats results in the dilation of both pupils with effects being greater on the ipsilateral side [170]. Therefore measuring pupil size under varying conditions can be informative when trying to assess central sympathetic and parasympathetic activity as outlined in Table 1 [171].

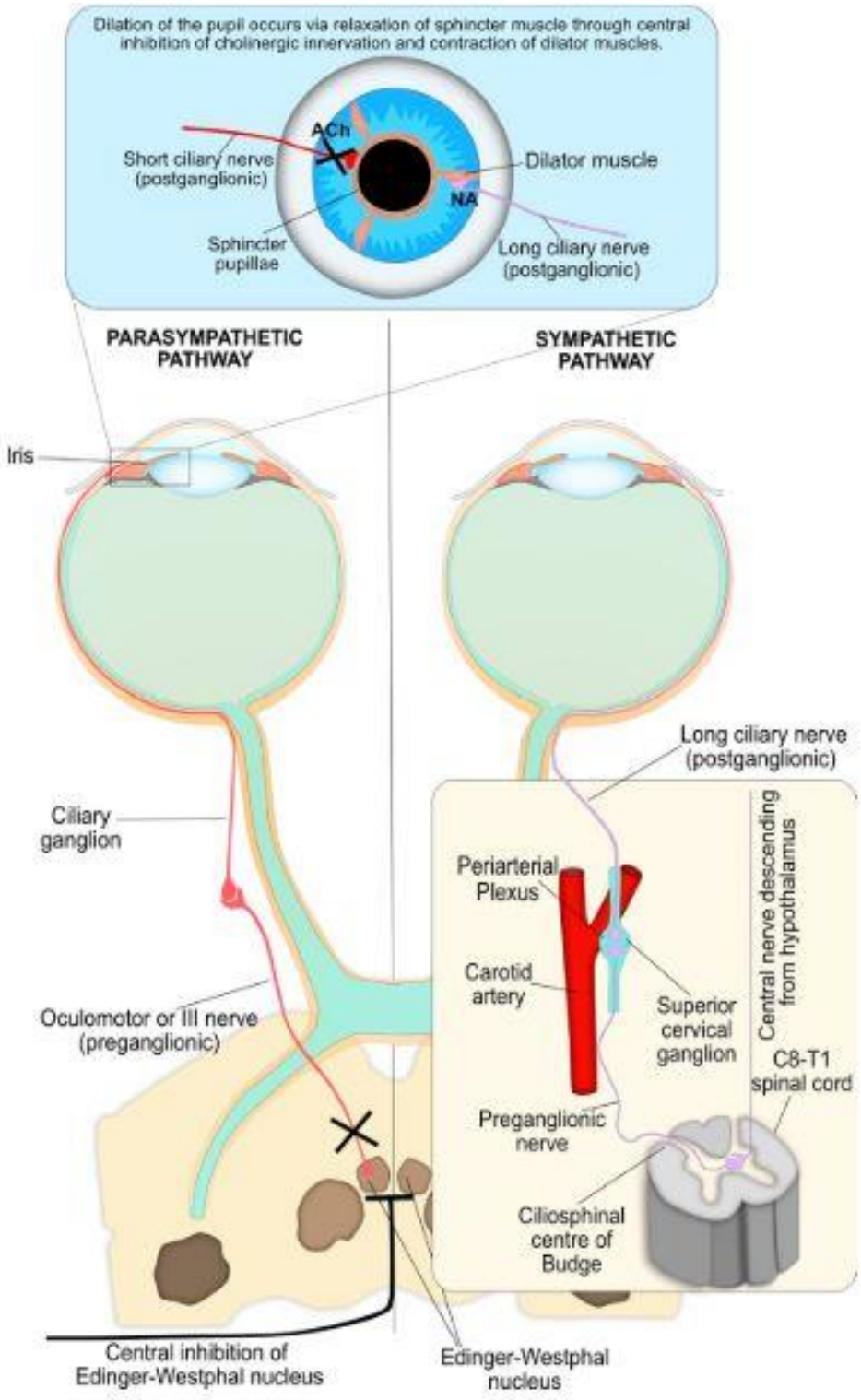


Figure 5. Illustration to show the parasympathetic and sympathetic pathways of pupil dilation.

Image obtained from Hall et al., 2018 [169]

Pupil dilation occurs by either noradrenergic inhibition of the Edinger-Westphal nucleus (via the Locus Coeruleus) preventing sphincter muscle contraction or by sympathetic innervation of the iris dilator muscle.

Table 1. Different pupillometry measurements and an interpretation of sympathetic vs. parasympathetic innervation

Condition	Description	Measurement	Results
Resting pupil diameter	Average pupil size measured at rest/baseline	Size of pupil (mm)	Decrease in resting pupil size = sympathetic deficit
Darkness Reflex	In ambient light at rest, switch off illumination for 1 second. Pupil dilation in response to darkness is recorded.	Amplitude (mm) - difference between baseline pupil size and maximum dilation	Increase = increase in sympathetic activity
		Maximum dilation velocity	Increase = increase in sympathetic activity
Light Reflex	In dark at rest, light flash at different illuminance levels for 200ms. Pupil constriction in response to light is recorded	Amplitude (mm) - difference between baseline pupil size and maximum constriction (mm)	Increase = increase in parasympathetic activity
		Maximum constriction velocity	Increase = increase in parasympathetic activity
		Latency (ms) - time between onset of stimulus and onset of response	Increase = sympathetic deficit
		75% recovery time (ms) - time to reach 75% size after the stimulus has ended	Increase = sympathetic deficit

Sympathetic activity influences the recovery time of the pupil response to the light stimulus in the light reflex condition and the measurements of the darkness reflex condition. The resting pupil diameter is smaller and the recovery time longer after a light flash is prolonged in older versus younger subjects suggesting a decrease in sympathetic innervation [171]. In addition, pharmacological studies show sedatives cause a smaller resting pupil size [172]; Yohimbine which increases noradrenergic activity, results in an increase in resting pupil size and; Clonidine, which decreases LC activity prolongs the recovery time after a light flash stimulus [173].

Pupil diameter can also be an indicator for level of arousal [174] and phasic activation of the LC in monkeys is related to task engagement and changes in pupil size [175,176]. In humans, better performance on tasks was associated with greater baseline pupil diameters and greater task-evoked pupil dilations indirectly indicating phasic activity of the LC and task engagement [177] [178].

Additionally pupil diameter correlates with BOLD (blood-oxygen-level-dependent) fMRI signal in an area corresponding anatomically to the LC [179].

1.2.3.4 NA and inflammation

Microglia have a high expression of adrenoreceptors. NA has an anti-inflammatory effect, regulating cytokine production in astrocytes and microglia including the suppression of proinflammatory cytokines such as IL-1, IL-6, TNF-α and inflammatory nitric oxide (iNOS) [180-182]. NA has also been found to affect the motility of processes in both activated and resting microglia[183] and is thought to reduce oxidative stress [184]. Reduction in LC activity and NA concentration is predicted to be associated with increased inflammation.

1.2.3.5 NA and Memory

Fusiform cells in the LC project to the hippocampus [185,186] and NA may therefore also have a role in memory formation. When doing a learned maze task, rats who received low level stimulation of electrodes in their LC beforehand made fewer errors suggesting NA plays a role in memory and attention [187]. Furthermore, this effect could be blocked using propranolol, a β-adrenoceptor antagonist [188].

It is difficult to solely affect LC neurons pharmacologically in humans or animals to determine their function. One way is to use DβH knockout mice which are unable to produce NA. DβH knockout mice have altered serotonin and dopamine signalling implying that the LC has a role in modulating other neurotransmitter systems besides NA [189,190].

The role of the LC is varied with dysfunction being connected to a range of disorders including REM sleep disorder, schizophrenia, anxiety and depression [191,192]. However, it has received considerable attention for its role in Alzheimer's Disease.

1.3 Locus Coeruleus and Alzheimer's Disease

1.3.1 Neuronal Loss

AD is associated with a significant amount of pathology in the LC. Firstly, there is a significant decrease in the number of LC neurons in people with AD compared to people of a similar age[117,193]. In a post-mortem study of 86 AD brains, there was a greater loss of cells in the LC than in the nucleus basalis [194] which has an established link with AD and is a target for the current licensed treatments for AD; acetylcholinesterase inhibitors. However not all studies have replicated this finding when comparing neuronal loss in these two areas [132].

Studies of LC cell counts using either tyrosine hydroxylase immunohistochemistry (IHC) or by counting cells that contain neuromelanin pigment, show an average of 60% reduction in cell number in people with AD compared to age-matched controls [115,131,195,196]. Additionally LC cell degeneration correlates with the duration and severity of AD [115,197] and is evident at the prodromal (MCI) stage [132]. One study found a 30% reduction in LC neurons in people with aMCI compared to controls with a further 25% reduction in LC neurons in people with aMCI compared to those with AD and greater cell loss correlating with greater cognitive impairment as measured by tests of memory, speed and visuospatial ability [113]. This early, stepwise deterioration of the LC suggests it could be a reliable marker for diagnosing AD, if it could be measured *in vivo*. Abnormalities in the appearance of LC cells, such as having swollen somata or shorter and fewer dendrites [198], as well as reductions in nucleolar volume and cytoplasmic RNA [193] are also detected in AD samples.

However LC neuronal loss in AD can be highly variable between individuals, with one study showing losses between 3.5% and 87.5% [198]. There is also a considerable reduction of LC cells in other diseases/conditions, for example Parkinson's Disease (PD) [194,198], Downs Syndrome [115] and AD with co-morbid depression [199], but not vascular dementia [196,200]. Some studies have even found LC cell loss to be more pronounced in PD than AD [194]. It is difficult to ascertain from the literature how early in the disease course of PD, LC neuron loss occurs. A recent review found that no studies of LC loss in PD employed stereological methods, and many did not specify the disease stage of PD or what type of PD the sample was from as this classification has come about after these studies have taken place [201]. Degeneration of the SN is still considered to be a more sensitive marker for PD [115]. Nevertheless, these findings have implications for the specificity of using the LC as a marker for AD diagnosis.

It may be that the pattern of LC cell loss distinguishes AD from PD. Thus although the length of the LC is shorter in both PD and AD compared to controls [198], in PD the greatest neuronal loss is in the caudal part of the LC [115]. In AD the pattern of cell loss is more variable, but with more prominent loss in the rostral [108,114] and middle portions of the LC [199,202] with the caudal neurons remaining relatively intact. This follows the pattern of cell loss that has been reported in typical aging, though is much more dramatic. It is also logical that the rostral and middle parts of the LC would be more significantly affected because these areas project to the temporal and frontal cortex and this correlates with the pattern of cerebral atrophy and associated cognitive deficits seen in AD. In a longitudinal study, 150 people over the age of 55 without a dementia diagnosis underwent regular assessments of cognitive function to measure cognitive decline over time until death. At post-mortem, it was found that a slower rate of cognitive decline was correlated with

increased density of the monoaminergic neurons in the LC, SN and DRN [203]. Additionally many AD patients report a loss of smell [204] and neurons of the LC project directly to the olfactory bulb.

One question that is now being asked is why the LC is so vulnerable to cell loss. One study looked at uptake of metallic mercury in someone who injected themselves with mercury before committing suicide and found heavy concentrations of mercury in only two regions, LC neurons and corticomotor neurons, with over 70% of LC neurons being affected [205]. It is thought that toxicants more readily enter the LC because LC neurons are exposed to the capillaries of the CNS (more so than any other neuronal network). This is known as the LC toxicant hypothesis. [206]. Furthermore, the LC is also located adjacent to the trigeminal ganglion and both projects to and receives input from the trigeminal nerve (based on animal studies) [207]. Recently neurodegeneration of the trigeminal nerve following tooth extraction in AD mice has been associated with cell loss in the LC, providing another mechanism by which LC neuron loss occurs in AD [208]. Additionally, it has been suggested that the nuclei's poorly myelinated neurons make them less resistant to oxidative stress and therefore more susceptible to AD pathology [27].

LC neuronal loss also correlates with the number of NFTs seen in the cingulate and temporal cortex [197] with one study demonstrating an 8.4% decrease in LC volume with each increase in Braak stage [114].

1.3.2 Amyloid Pathology in the LC

Although studies correlate increased LC cell loss with an increase in cortical Aβ plaques in AD [197], amyloid pathology is not typically seen within the LC or brainstem until the later stages of AD progression [209] as shown in Figure 6. One study identified dense core plaques within 62% of the LC cells counted in AD samples. Unfortunately the severity of dementia was not recorded, though the average duration of dementia for the samples was 9 years and the average age of disease onset was 66 years, suggesting that these samples were not from mild dementia cases [36]. The link between LC neuronal loss and Aβ deposition is unclear.

1.3.3 Tau pathology in the LC

Both MCI and AD patients show more NFTs in the LC (as well as the other brainstem nuclei), compared to those without cognitive impairment [210,211]. The amount of tau pathology in the LC correlates with dementia severity [199] and continues to accumulate throughout the disease process[212], but is most prominent in the middle of the LC where cell loss is reported to be most pronounced [199,213].

A recent hypothesis states that tau pathology originates in the LC and other subcortical nuclei [27]. One study examining 2332 brains aged 1 year to 100 years using the antibody AT8 to stain for p-tau in 100µm sections [214] in order to map the pattern of abnormal tau pathology that occurs prior to Braak stages I-VI. Only the very young brains had no abnormal tau, and 58 out of the 2332 samples showed p-tau in the absence of any cortical abnormalities. Of these 58 samples, p-tau was always present in the LC, often only found in the LC and was present from an early age i.e. before 30 years [133]. Another study found approximately 30% of cognitively normal brains younger than 50 years had p-tau in the LC even when using this 7µm thick sections [215]. As these are cross-sectional studies, it is not possible to assess whether early p-tau accumulation in the LC is an indicator for predicting who would go on to develop AD. However, it is clear that the LC is particularly vulnerable to early tau pathology and this has led to the addition of Stages a, b and c to the Braak staging system which describe the accumulation of hyperphosphorylated tau, firstly in the neurites of the LC (a), then in LC cell bodies (b), before affecting other subcortical nuclei (c), all precluding the cortical accumulation of tau observed in Stages I-IV [216]. This occurs both before cognitive impairment and the onset of LC cell loss which is not significant until Braak Stage III [212,217]. However, these studies cannot determine whether this early tau accumulation affects the function of the LC cells. Additionally, the delay from p-tau first being observed in the LC, to LC cell death decades later is not understood. Does a threshold of p-tau accumulation have to be met in order to trigger cell death or is there another mechanism at play?

Interestingly one study, examining tau and amyloid pathology in all brainstem nuclei including cranial nerve nuclei found that the only cranial nerves to be affected in AD were the dorsal motor nucleus of the vagus nerve, Edinger Westphal Nucleus, nucleus tractus solitarius and nucleus ambiguus which are all associated with the parasympathetic nervous system as is the LC [36].

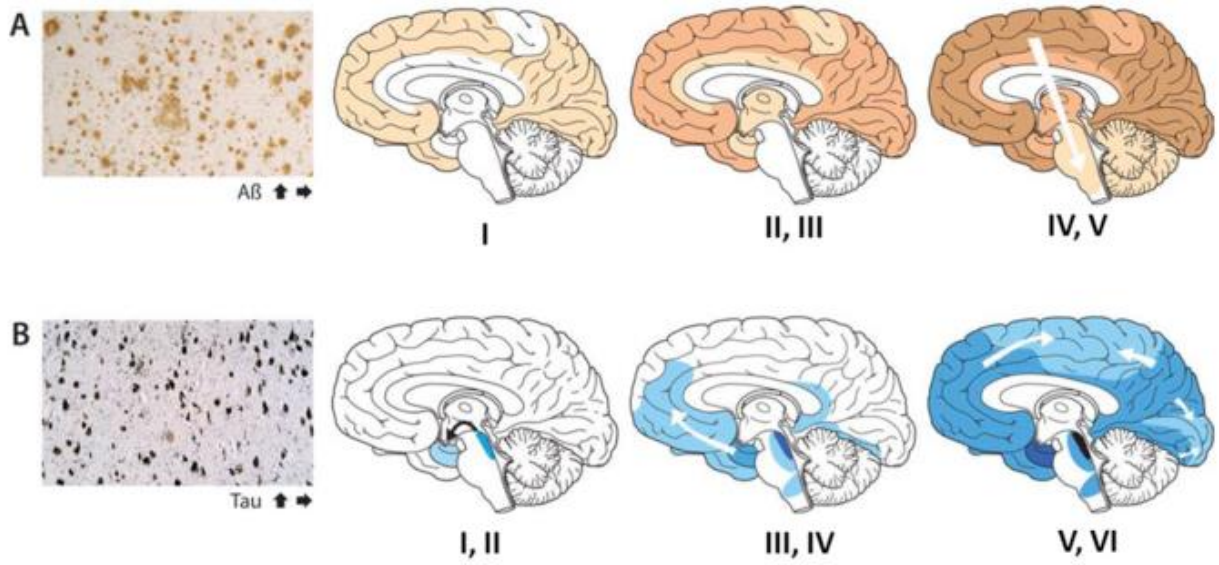


Figure 6. Location of A) amyloid plaque deposition and B) NFT progression as disease severity increases.

Image taken from Jouanne et al., [218] and adapted from data by Braak and Thal [133].

Several studies have identified other subcortical nuclei in the isodendritic network, such as the DRN to show hyperphosphorylated tau around the same time or just after the LC, again decades before disease onset [14,219]. These nuclei all project to the transentorhinal and entorhinal cortex, which are the first cortical regions to see high numbers of NFTs and undergo extensive neuronal loss during AD [27]. One theory supposes that tau pathology originates in the LC (and other brainstem nuclei) and spreads through the cortex via their diffuse projections because p-tau has a chronological, predictable topographic pattern. The prion hypothesis suggests abnormal tau proteins are prions which cause nearby normal tau to become hyperphosphorylated, therefore causing a spreading of misfolded tau protein throughout the brain, like an infection. This hierarchical pattern of spreading of tau pathology has been demonstrated in the hippocampus where areas that are affected are connected via projections from previously affected areas [220]. As the LC cells innervate so much of the cortex they could easily enable the spread of pathology if it is able to move trans-synaptically from neuron to neuron [217].

Injections of p-tau into the hippocampus of young PS19 mice, which over-express mutant tau, resulted in increased tau in the LC [221]. This suggests that the spread of tau from the LC to the hippocampus can occur both ways lending weight to the idea that tau pathology spreads from one region to another. However, the mechanisms behind the release of tau across the synapse are not known. Although PET ligands for tau exist, it is not yet possible to study p-tau pathology within the LC in humans *in vivo* or to measure the functional consequences of this because of the

tiny size of the LC, though as imaging techniques improve this may be something that could be explored in the future.

1.3.4 Noradrenaline and Alzheimer's Disease

It follows that with the degeneration of LC neurons there will be a corresponding effect on the concentration of NA in the brain, which is predictably lower in those with AD compared to controls [222] particularly in the temporal lobes [131,196,223-225] and shows direct correlation with LC cell count [197], although lower NA levels have not always been observed [226]. A small study comparing NA loss in 8 different brain regions including the hypothalamus, caudate and hippocampus showed this to be specific to AD as subjects with multi-infarct dementia or depression were not affected [196,225]. This reduction in NA in the temporal cortex was observed *in vivo* in 5 individuals with AD that underwent a diagnostic craniotomy [227]. Furthermore, one PET study showed reductions in NET in the thalamus of post-mortem AD brains compared to controls which correlated with increased Braak stage [228]. CSF levels of NA are reduced in AD, PD and multiple system atrophy and lower levels of MHPG (3-Methoxy-4-hydroxyphenylglycol) suggesting NA degradation, were linked to longer duration of illness and lower cognitive scores [229].

Autophagy has been shown to be accelerated by stimulation of β2-ARs [230]; a lack of NA may therefore mean cells are less able to repair and remove abnormal tau [217]. Additionally NA has anti-inflammatory influences on microglia which have been exposed to fibrillar Aβ [231]. NA inhibits IL-1 secretion by microglia. As mentioned earlier IL-1 is linked with APP production and therefore if NA levels are reduced then IL-1 is not being sufficiently suppressed which may contribute to disease progression [181].

Cultured cortical and LC cells are protected (though not fully) from Aβ induced cell loss by β-adrenergic stimulation by NA which increases expression of brain-derived neurotrophic factor BDNF. Therefore a decrease in NA concentration may remove this neuroprotective effect [232,233].

Furthermore, LC cell loss and abnormalities in noradrenergic innervation of the brain may explain the behavioural and psychological symptoms also associated with AD such as depression, sleep disturbance and anxiety [234]. Reductions in NA in AD would also be expected to cause dysfunction in the sympathetic nervous system and is thought to be the cause of the reduced darkness reflex of the pupil in response to light in those with AD [235].

Though the role of decreased cortical NA concentration on increasing inflammation is robust, no-one has examined post-mortem human tissue to see if the inflammatory changes observed

elsewhere in the brain also occur in the LC; moreover, it also remains to be seen whether LC cell loss is associated with inflammatory changes in the temporal cortex.

1.3.5 Animal models

Transgenic mouse models have been used to study the role of the LC in AD. Tg2576 and PDAPP mice both over express APP and 5xFAD and APP/PS1 mice additionally overexpress presenilin-1, all resulting in an overproduction of Aβ, whereas PS19 and P301S mice have mutations which cause over expression of tau. DSP4 N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine) is a neurotoxin which accumulates in noradrenergic neuron terminals and causes an almost complete loss of NA to brain areas that are innervated selectively by the LC[236,237]. Therefore DSP4 injections have been used to study the loss of the LC-NA system in these animals. However, LC cells do not only synthesise NA and therefore, in order to understand the effects of LC loss, DβH knockout mice and Ear2 -/- mice are used. DβH knockout mice have LC neurons that are unable to synthesise NA but are otherwise unaffected in terms of neuron number or number of projections. Ear2 is an orphan nuclear receptor expressed in LC precursors and is required at an early stage for LC development. Ear2 -/- mice have a severe loss (over 70%) of LC neurons, primarily those that project to the hippocampus and neocortex as well as reduced cortical NA[238]. A summary of the murine studies describing LC degeneration are detailed in Table 2.

Aged APP/PS1 mice have 25% less LC cells than age-matched wild type mice whereas SN dopaminergic cells are unaffected [75,239,240]. Likewise, gene expression for DβH and TH is significantly reduced in 5xFAD mice compared to wild-type mice [241]. NET immunostaining is reduced and there are fewer TH positive axons detected in the prefrontal cortex, the hippocampus and spinal cord of APP/PS1 mice than in age-matched wild-type mice, although interestingly this was not associated with a reduction in NA levels as measured by ELISA [75]. The surviving LC neurons also have larger cell bodies and short thick broken dendrites when compared to wild-type mice [239,241] all of which is similarly seen in *post-mortem* studies of AD in humans [198]. At 8 months the volume of the LC in Tg2576 mice is reduced. Surprisingly, in one study an initial increase in positively stained DβH cell density between 6.5 to 8 months was also observed, suggesting an initial compensatory strategy to the loss of LC cells [242]. However one study using PDAPP mice showed no change in the number of LC cells compared to wild type mice [243].

DSP4 injections in APP/PS1 mice which effectively causes LC degeneration, results in greater accumulation of Aβ in brain areas that the LC projects to [244,245] with one study showing a 5-fold increase in the number of Aβ plaques seen at 9 months in APP mice with DSP4 injection compared to those without DSP4 treatment [246]. Recently DSP4 injections administered to aged rhesus

macaques also resulted in increased cortical Aβ deposition when compared to saline treated animals, as well as increases in APP and BACE1 in the temporal cortex, suggesting that NA depletion alters APP processing of Aβ [247].

Injections of DSP4 into both rats and APP/PS1 mice results in a 70-80% reduction of NA in the forebrain as well as a reduction in hippocampal NA concentration [246,248]. Furthermore, DSP4 injections in both APP/PS1 and P301S mice are also associated with greater cognitive impairments in spatial memory as measured by the Morris Water Maze task [244,249] and ablation of the LC resulted in rats making more working memory errors [248] correlating with reduced proliferation of noradrenergic cells in the dentate gyrus of the hippocampus [250]. APP/PS1 mice crossed with DβH -/-, or Ear2 -/- mice show greater impairment in spatial memory tasks and long-term potentiation compared to APP/PS1 mice alone [251,252]. This suggests a role for NA specifically in memory loss in AD, rather than the LC's involvement in other neurotransmitter systems. Furthermore, when L-DOPS was administered to increase NA levels some improvement in spatial memory task performance was observed, though these mice still did not do as well on the task as APP/PS1 mice without NA depletion [251,252].

Additionally in rats, DSP4 caused decreases in tight junction proteins in the endothelium of brain vessels affecting the permeability of the blood brain barrier. Reductions in NA potentially interferes with the clearance of Aβ and other toxins [253] suggesting another route by which an impaired LC could participate in AD onset.

Because of its anti-inflammatory effects, decreased NA levels are expected to affect the regulation of pro-inflammatory cytokines which is then theorised to cause further noradrenergic cell loss in the LC resulting in a negative feedback loop [254]. Animal models support this hypothesis, for example injecting rats with DSP4 to induce LC degeneration results in greater expression of iNOS, IL-1 and IL-6 [246,255]. Both 5xFAD mice and P301S tau mice show increased markers of microglial and astrocyte activation in the LC compared to wild type mice and do not show increased inflammatory markers in the SN [241,249]. When injected into APP23 mice DSP4 caused greater glial inflammation than when injected into wild-type mice. Additionally LC projection areas also showed elevated levels of glial inflammation [245]. In mice, NA maintains the clearance of Aβ by microglia and also suppresses Aβ induced cytokine production [231,246,256] and interestingly increasing NA concentration in mice slowed the rate of neurodegeneration [257]. Overall, these animal studies clearly suggest that LC degeneration and a reduction in NA, participates in the progression of AD and accumulation of Aβ. Though this remains to be confirmed in humans, NA might be a potential target for pharmacological treatment.

Table 2. Summary of studies using transgenic mouse models to explore Locus Coeruleus degeneration in Alzheimer's Disease

Reference	Animal model	Method	Summary
Liu et al., 2013[239]	APP/PS1 young 5-6m mice (*n=6*), APP/PS1 aged 16-17m mice (*n=6*), vs age-matched wild-type mice	IHC for TH+ neurons on 25µm thick serial coronal sections of LC	Aged APP/PS1 mice had 23% fewer LC cells than both wild-type and young APP/PS1 mice. Remaining LC cells had large cell bodies with short, thick processes
O'Neill et al., 2007[240]	APP/PS1 young 3-6 mice (*n=6*), APP/PS1 aged 16-23m mice (*n=7*) vs age-matched wild-type mice	IHC for TH+ neurons in LC, Substantia Nigra and Ventral tegmental area (VTA)	Aged APP/PS1 mice had 25% fewer TH+ neurons in the LC, but no change in the SN or VTA
Guérin et al., 2009[242]	Tg2576 mice (*n=29*) vs wild-type mice (*n=22*)	Injected at 6.5-7m with BrdU, marker for neurogenesis, 10 Tg2576 and 10 wild-type mice killed immediately, rest killed 45 days later. IHC for DBH+ neurons.	Reduction in LC volume between 6.5 to 8months in Tg2576 mice compared to wild-type mice. At 8m DBH expression increased suggesting possible compensatory mechanisms.
German et al., 2005[243]	PDAPP mice aged 23m (*n=5*) vs age-matched wild-type mice (*n=5*)	IHC for TH+ neurons in LC	No differences in number of TH+ neurons, counted from rostral to caudal
Jardanhazi-Kurutz et al., 2010[244]	APP/PS1 mice vs wild-type mice treated with either DSP4 injections or saline	injections started at 3m and then monthly after. Behavior testing at 4, 6, 12m and killed at 4.5, 6.5 and 12.5m. IHC for NET-IR in medial LC compared to cortical NET-IR and BCA assay to determine Aβ protein concentration	In DSP4 treated mice, LC NET-IR and Cortical NET-IR was reduced. At 6.5m Aβ levels were higher in DSP4-treated (NA depleted) APP/PS1 mice compared to saline-treated. Spatial memory tasks also more impaired in DSP4-treated groups.
Heneka et al., 2006[245]	APP23 mice vs wild-type mice treated with either DSP4 injection or saline	DSP4 injection/saline give at 10 months, animals sacrificed at 16months, IHC for TH+ neurons. IHC to examine glial activation using CD11b and GFAP	50-60% reduction in TH+ LC neurons and 70% reduction in cortical NA levels in DSP4-treated mice. Little glial activation in wild-type mice even when treated with DSP4. Some glial activation in APP23 mice which was greatly increased in DSP4-treated mice. Additionally, greater memory deficits in DSP4-treated APP23 mice compared to saline-treated.
Kalinin et al., 2012[241]	5 x FAD mice aged 4-5months vs age-matched wild-type	Treated with L-DOPS 3xper week for 1 month to increase NA levels. Behavior testing performed after treatment. NA levels measured. IHC for TH+ cells and GPAP on 35µm thick serial sagittal sections.	No differences in numbers of TH+ cells between 5xFAD mice and wild-type mice, but TH+ cells in 5xFAD were significantly bigger. Increased glial inflammation in 5xFAD vs wild-type mice. L-DOPS treatment improved performance in the Morris Water Maze task and reduced glial inflammation.

Aβ: Amyloid-beta; DBH: Dopamine Beta Hydroxylase; DSP4: N-(2-chloroetyl)-N-ethyl-2-bromobenzylamine; IHC: Immunohistochemistry; TH: Tyrosine Hydroxylase; L-DOPS: L-threo-dihydroxyphenylserine; NET: Noradrenaline Transporter

1.3.6 Neuromelanin, Iron Deposition and Alzheimer's Disease

NM is thought to be neuroprotective as it has a strong affinity to metals such as iron, copper and zinc, containing them in an insoluble, inactive state within the LC cells and preventing these toxins accumulating in other places [142,143]. Iron is the most abundant metal in NM and therefore it has been suggested that NM has a role in protecting the brainstem from oxidative stress. The NM in the SN may play a greater role in this than the LC as it has a greater iron content; the NM in the LC has a greater copper content because of the DβH [142]. NM loss in the SN in PD is associated with increased iron levels which correlate with disease severity [258].

NM loss is associated with LC cell loss in AD [196], though it is mainly NM loss in the SN occurring in PD that has been studied [259,260]. It has been theorised that as cells containing NM degenerate, the NM is released into the extracellular space [143] and because it is insoluble it stays there for a long time which could act as a pro-inflammatory stimulus triggering cytokine secretion. Microglial cell culture experiments show that when NM is added there is a dose-dependent overexpression of the proinflammatory cytokines TNFα, IL-Iβ and iNOS [261] and similarly when added to rat microglial cultures, there was an increase in TNF-α, IL-6 and nitric oxide [262]. When dopamine neuron and microglia co-cultures are exposed to NM there is additional neuronal loss, which only occurs when microglia are present, i.e., the NM is not toxic to the dopamine cells when there are no microglia present. Therefore microglial activation is thought to exacerbate neuron degeneration in the presence of NM [263]. Timelapse videos show microglia are able to phagocytose NM granules that ranged in size from 2 to 14μm. Interestingly microglia that were aged in culture for approximately 1 month in vitro were less efficient in phagocytosing NM than those that were 10-20 days old [261]. In cell cultures the microglia are exposed to NM rapidly in a way they wouldn't during SN/LC degeneration and so this may not be reflective of what occurs in the human brain.

There are very few human studies that have explored how extraneuronal NM affects the surrounding neurons. Most accounts regarding the presence of extraneuronal NM in either the SN [264-266] or the LC [196,199,200] are anecdotal with NM not being specifically stained for. NM deposits have not reportedly been observed in either the SN or LC in younger brains aged under 30 [133]. To my knowledge only one study has attempted to quantify the amount of extracellular neuromelanin present in the SN, using a rating scale from 0-absent to 4-frequent to compare NM deposition with age. Extraneuronal NM deposits were only observed in elderly brains and additionally correlated with staining for activated microglia [267]. There have been no studies which quantify extraneuronal NM in the LC either in ageing or in AD or correlate it with disease severity.

Additionally in post-mortem studies microglia have been observed to gather around NM deposits and to contain NM particles, both in the SN [265,267] and in the LC [108,196] again suggesting its toxicity, but how and if NM is then degraded is not known [139]. It is also unclear what happens to the metals that were contained within the LC cells as they degenerate.

When human NM was injected into the rat or mouse cortex it causes a strong inflammatory reaction in the form of increased microglial activation evidenced by Iba1 staining in both areas[260,268]. Additionally when NM is injected into the SN, it is associated with approximately 40% loss of dopamine neurons with areas that have the greatest staining for microglia also having the most cell loss [261]. This shows how a positive feedback loop caused by extracellular NM could accelerate the loss of neurons in the SN [260] and therefore potentially also in the LC. Extracellular NM combined with the reduced concentration of NA is likely to result in a pro-inflammatory environment which may contribute to neurodegeneration.

A point of interest is that NM is only found in such high quantities in the human LC and to a much lesser extent in the LC of animals, and therefore this is a limitation of AD mice models when attempting to generalise findings to human AD. Another limitation when applying these findings to the LC is that NM is typically isolated from the SN or injected into the SN and similar experiments on the LC and noradrenergic neuronal cultures have not been studied. However, as NM in SN and LC is thought to be structurally similar and NA and DA neurons share similar features, it is likely that similar results would be witnessed in the LC.

To date there have been no human post-mortem studies which examine the relationship between extra-cellular NM and inflammatory markers within the LC.

1.4 Neuromelanin-sensitive Magnetic Resonance Imaging

MR scanners have a strong external magnetic field; all the protons in the tissue being scanned align with this magnetic field, known as B_0. When a radio frequency (RF) pulse is applied intermittently, it temporarily disrupts the field and all of the protons relax back into their resting positions emitting RF energy. These signals are measured and transformed into intensity values displayed as a range of greys. The time it takes for the proton to align back to the magnetic field is known as its relaxation time and different tissues have different relaxation times. Tissues with short T1 times e.g., fat, appear as bright hyperintense values whereas CSF takes a long time to re-align, not fully re-aligning before the next RF pulse and therefore appears black. In a T1 weighted image the time between each RF pulse, known as the repetition time or TR, is short. Using MRI to look at the structure and connectivity of the brainstem nuclei *in vivo* is challenging because of their small size and complex organisation. However, NM has paramagnetic properties due to its

binding of iron and copper, giving it a short T1 relaxation time [269]. This means the LC and SN can be visualised using T1 weighted MRI, using a 3Tesla or greater scanner, as hyperintense areas[269,270]. Additionally, the combination of NM with macromolecules i.e., proteins and lipids, is also thought to enhance the signal of NM due to Magnetisation Transfer (MT) effects [271,272]. It is likely that the signal comes from a combination of T1 shortening and MT effects [136,269] and a thorough understanding of what causes the contrast is still lacking [273,274]. Imaging protocols designed to enhance this signal have been termed neuromelanin-sensitive MRI (NM-MRI).

Though most have used a fast/turbo speed echo sequence (FSE/TSE) acquisition protocol [275-279] , the LC and SN have also successfully been imaged using FLASH imaging [280] or Gradient Echo sequence (GRE) with MT contrast [281,282] with proponents of both arguing that this enables better visualisation of the LC. At the time of commencing the clinical project, only one study had directly compared TSE to GRE with MT contrast and found no differences in signal contrast in the LC but found GRE with MT contrast resulted in a lower specific absorption rate [282]. Additionally even when the same type of scan is used, different parameters are still set to obtain the images so there is considerable variation between acquisition methods and there is still much debate surrounding the optimum method for imaging the LC [283].

The LC signal on NM-MRI correlates with LC volume post-mortem [271,284] suggesting this is a reliable method to explore LC signal in vivo. Generally the signal intensity of the LC is stronger in the middle aspect of the LC [276,280,285] and typically researchers identify the axial slice of the brainstem which is approximately 7mm below the inferior colliculi to measure the intensity values of the LC [275,286]. The signal intensity of the LC and SN are typically calculated by using the peak signal intensity of the area and comparing it to a relevant reference area to calculate a contrast ratio (CR). For the LC this reference area is typically the pons tegmentum (PT). The most frequently used formula for calculating the CR is (signal intensity of LC – mean signal intensity of the PT)/mean signal intensity of the PT, though this varies between studies with some dividing by the standard deviation of the PT [279]. It has been suggested that the PT is itself subject to age-related decline and so may not be the most reliable reference region [287], though alternative reference areas are yet to be found. Some authors have used automated methods to delineate the LC using a probabilistic reference atlas [287-289]. Whilst some have used a semi-automated method to identify the location of the LC [282], most have used manual techniques, instead using multiple raters completing blinded analysis, and averaging 3 calculations to overcome any attributable human error [275,284,285]. Differences in acquisition methods as well as different methodological approaches to analyse the LC signal, make it difficult to compare studies.

One limitation of NM-MRI is that is has low spatial resolution compared to the size of the LC resulting in a long acquisition time. Additionally results are confounded by noise from the cardiac system which pulses through the brainstem if imaged at 3Tesla or below [290]. As known from post-mortem studies there is considerable individual variation in LC size [114], and therefore bigger sample sizes are needed to assess differences in LC signal between patient groups and healthy controls. Furthermore, by measuring peak signal intensities rather than looking at overall signal or looking at differences in different aspects of the LC it is possible that any differences between patient groups and healthy controls are masked. However, the protocol does have good test-retest reproducibility [280,289,291]. Nicotine (and therefore smoking) can enhance the signal of the LC as nicotine has been shown to increase LC firing rate [292].

In healthy participants, the signal of the LC measured using NM-MRI increases with age until approximately age 60 and then slowly decreases [277,283] following the same inverted U pattern of NM accumulation that is seen in post-mortem studies [116,134]. However some studies have not observed any age-related differences in LC signal [280,293], whilst others suggest LC signal increases with age, though this is only demonstrated when comparing older adults over the age of 60 to younger adults rather than including mid-life controls or studying changes in LC signal across the lifespan [286].

When examining the rostrocaudal extent of the LC, one large study of over 600 participants found there to only be a reduction in signal in older adults in the rostral portion of the LC [283]. Similarly when cell loss has been reported in older adults in post-mortem studies it was the rostral part of the LC that was affected [116,120]. However, like the post-mortem studies, participants in the NM-MRI study were not followed up to see if they developed neurodegenerative disease or to assess their cognition. On NM-MRI, LC signal is positively correlated with cognitive reserve, measured by looking at years of education, occupation and verbal intelligence [286] and more recently memory performance across a variety of tasks [294] including recall of emotionally salient events [293]. This again mirrors post-mortem studies that have found cognitive performance to be associated with LC integrity [203]. Interestingly the rostral LC signal correlated better with memory performance [294] which is unsurprising as the rostral part of the LC is known to project to the hippocampus. Overall, there have been mixed findings when it comes to age effects in both imaging and post-mortem studies and the uncertainty around this suggests it is something to be considered when looking at LC signal [277].

NM-MRI has been utilised to explore differences in NM concentrations in various disorders and is supportive of current monoamine dysfunction hypotheses for these. For example, in schizophrenia which is thought to be caused by hyperactivity of the dopaminergic system, the

signal intensity of the SN is significantly higher compared to healthy controls [276]. In depression which is known to be caused by dysfunction of the noradrenergic system, the LC signal is lower in the rostral and middle portions of the LC when compared to healthy controls [276]. Participants with REM sleep disorder showed reduced signal intensity in the caudal part of the LC [295,296] corresponding to what has been demonstrated in post-mortem studies.

However to date NM-MRI's main application has been to explore differences in the signal from the SN and LC in Parkinson's Disease [279]. Both areas show a significant reduction in signal intensity [284,288,297], though reductions in the signal of the SN correlate more closely with PD diagnosis, duration and severity [278,288,298] again in line with post-mortem evidence. Furthermore, the signal of the SN correlates with manual counts of NM containing neurons which again supports this as a useful technique to look at NM concentration *in vivo* [299].

At the time of commencing the clinical part of this project only two studies using NM-MRI in populations with AD had been published. One found no significant differences in LC signal intensity between AD and healthy controls (although this study only had 6 participants) [298], whereas the other study found a significant reduction in signal in AD patients [275]. LC signal was also reduced in those with MCI compared to controls, however these changes were not predictive of who would go on to convert to AD. Unfortunately, this study did not employ age and gender matched controls and the healthy control group were significantly younger which may have affected the results as the LC signal has shown age-dependent variance in some studies. Following a review of all NM-MRI and LC papers it was determined that more studies are needed to confirm whether a signal intensity decrease in the LC is observable in AD and to see if NM-MRI is a viable early-stage biomarker [279].

More recently, three further studies have also found LC signal intensity to be reduced in AD. One study focused on identifying the best method for differentiating AD participants from healthy controls by analysing 5 slices covering the rostrocaudal extent of the LC [300]. They found that the slices 7.5mm and 10mm below the inferior colliculi was where the LC signal was strongest and best separated the two groups. However, they had a small sample size of only 10 participants in each group (though controls were age and gender matched) and AD participants varied greatly in degree of cognitive impairment with MMSE scores ranging from 10 to 26. In the two other studies, one showed LC signal to be reduced in those with atypical AD i.e. logopenic progressive aphasia (LPA) and visuospatial deficits as well as in typical AD [301] and another compared LC signal in AD participants to those with MCI or subjective cognitive impairment and found signal in the AD group only to differ from controls [302] as well as being reduced in those with atypical AD. However LC signal didn't correlate with dementia severity as measured by MMSE or with cortical

amyloid load as measured using PET imaging [301] or tau or amyloid CSF biomarkers [302]. Again, these studies did not use age and gender matched controls. Another limitation of these studies using AD populations is that they included participants who were taking medications that affect the noradrenergic system e.g., serotonin and norepinephrine reuptake inhibitors which may alter the LC signal.

It has been shown that LC volume measurements are highly reproducible when repeated across multiple scans in young people [291] and this would suggest that if there are changes in LC signal over time in AD that these could be studied longitudinally and non-invasively. However detection of LC signal is still in its infancy and has hardly been explored in the context of AD. Whether this tool could be used as a biomarker for AD and how early any changes can be detected remains to be answered.

Chapter 2 Hypothesis, Aims and Experimental Approach

2.1 Overarching Hypothesis

The overarching hypothesis for this thesis is

Neuropathology in Alzheimer's Disease originates in the Locus Coeruleus.

To investigate this hypothesis, the study was split into two separately funded projects with two separate hypotheses.

2.2 Hypothesis 1

Neuromelanin-sensitive MRI can be used as a biomarker to detect LC signal intensity reduction in AD patients in vivo and will correlate with indirect measures of LC activity

The pathology associated with AD, namely neuronal loss, extracellular Aβ deposition and intracellular aggregation of p-tau occurs gradually over a period of decades and is present prior to the onset of cognitive symptoms. Post-mortem evidence shows that the LC undergoes significant, early neuronal degeneration during AD with hyperphosphorylated tau pathology now being hypothesised to originate in the LC and other subcortical nuclei. However current biomarkers for AD are unable to detect pathology before the onset of cognitive symptoms.

Recent advances in MRI technology have established the NM-sensitive sequence to be an accurate indicator of SN integrity, but detection of LC signal is still in its infancy and has hardly been explored in the context of AD. Whether this tool could be used as a biomarker for AD and how early any changes can be detected remains to be answered.

This study aims to optimise the NM-MRI protocol for visualising the LC using age and gender matched controls in order to explore whether signal differences in the LC can be detected between those with and without AD. Additionally, it aims to examine the link between LC signal changes and indirect physiological measures of LC activity e.g., blood pressure and pupil diameter which has not yet been explored. If imaging can be used in the detection of LC degeneration it could enable the LC to be studied throughout disease progression to better understand its role in AD.

2.2.1 Aims

1. To examine the feasibility of detecting signal intensity changes within the LC using a high-resolution turbo spin-echo neuromelanin-sensitive MRI technique in those with AD compared to those that are cognitively normal in a pilot study. If the data suggests this technique can distinguish AD from control participants, it will be used to inform a future large-scale study to see how early these changes can be detected.
2. To examine if LC signal intensity on NM-MRI is associated with peripheral inflammatory markers.
3. To examine how LC signal intensity on NM-MRI correlates with physiological measures of autonomic function and pupillometry measures.

2.2.2 Experimental Approach

A cross-sectional observational clinical study was conducted to investigate differences in LC signal using a high-resolution NM-MRI sequence in 12 participants with mild AD, 12 participants with moderate AD and 24 age and gender matched controls. All subjects completed a battery of cognitive assessments and, in those with an AD diagnosis their study partner was asked additional questions about the participant's behavioural and functional ability. Clinical data was obtained along with various measures of autonomic function including blood pressure changes and eye-tracking to record the pupil response to light. Blood samples were obtained to investigate peripheral cytokine levels. All subjects underwent a MRI using the NM-MRI sequence and all of the data obtained was correlated with LC signal intensity observed on the MRI. This was a pilot proof of concept study to examine the feasibility of detecting LC signal intensity changes using this technique with the aim of informing a larger future scale study which would include participants at earlier stages of the disease.

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

2.3 Hypothesis 2

Local inflammatory changes within the LC occur early in the disease process and are associated with LC neuronal loss and extraneuronal neuromelanin accumulation. Changes observed in the LC will be associated with inflammatory changes and other neuropathological features of AD in the temporal cortex.

The role of inflammation in disease progression in AD is receiving more attention and changes in inflammatory markers in the cortex have been investigated somewhat, particularly in response to

Aβ plaques. However inflammatory changes within the LC and how these relate to changes observed elsewhere in the brain have been largely overlooked. In addition, the effects of LC degeneration in AD on extraneuronal neuromelanin deposits has not been quantified and the relationship between increased extraneuronal NM with inflammation in the LC has not been examined. This post-mortem study will aim to assess how LC cell loss is associated with inflammatory markers, both within the LC and an area of the temporal cortex the LC projects to. By assessing samples obtained at different stages of disease severity, the findings of this study may provide some answers on the timeline of events in terms of neuropathology and neuroinflammation in these two brain areas. Understanding these relationships may inform us further on whether the pharmacological elevation of NA would be successful in slowing disease progression and at which time-points it should be administered. If NA manipulation was able to delay AD pathology, then early detection and monitoring of LC degeneration *in vivo* using NM-MRI as a biomarker would be essential.

2.3.1 Aims

1. To examine the relationship of LC neuronal loss and disease severity with inflammatory changes, intracellular and extracellular neuromelanin deposits and AD neuropathological changes within the LC.
2. To examine the relationship of LC neuronal loss and inflammatory changes with cortical inflammatory changes and associated AD neuropathology.

2.3.2 Experimental Approach

This was a post-mortem study using immunohistochemistry (IHC) to look at a variety of markers of pathology associated with AD as well as markers of inflammation in human brain tissue. 60 cases each with two brain regions the LC and the temporal cortex were examined for this study and were stratified into 3 groups by Braak stage (0-II, II-IV, V-VI) as a measure of disease severity. Regions of interest were extracted from the immunostained tissue for each marker, for each case and the percentage of the protein load was calculated for each brain area. Differences in protein load both between groups and between brain regions were examined and markers of neuronal loss and AD-pathology were correlated with markers of inflammation.

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

Chapter 3 Investigating the role of the Locus Coeruleus using NM-MRI

3.1 Method

3.1.1 Ethics

This study was funded by Alzheimer's Research UK (ARUK-PPG2016A-6). The Chief Investigator (CI) for the study was Dr Ruihua Hou.

The study was registered with the University of Southampton Ethics Electronic Research Governance (ERGO, Ref: 23259). Ethical approval was provided by the North West Haydock Research Ethics Committee on 3rd October 2016, REC reference 16/NW/0675. Health Research Authority (HRA) approval was given on 28th December 2016. Subsequent local Southern Health NHS (SHFT) R&D approval was given on 10th January 2017 and University Hospital Southampton (UHS) NHS R&D gave approval on 31st January 2017, see Appendix C.

Study material including the protocol and patient information sheets and consent forms were prepared by myself and Dr Ruihua Hou. Once appropriate validated assessments were selected to quantify cognitive status and any behavioural difficulties, permission was sought from the authors where required to use them in this study. Case report forms (CRF) to capture the data were designed by myself; all study material is provided in Appendix C.

An amendment to the original protocol and participant information sheets (PIS) was submitted for ethical approval on the 8th September 2017 to include an additional cognitive test, the Montreal Cognitive Assessment (MoCA) and update the PIS to give more detail about the cold pressor test, one of the physiological measures of autonomic function. This was approved by the REC and HRA on 13th October 2017 and subsequent SHFT R&D approval was given on 23rd October 2017. A further non-substantial amendment to extend the recruitment end date from the 31st August 2018 to the 31st October 2018, was submitted on the 30th August 2018 and approved by the HRA on 31st August 2018.

The first participant was recruited on 17th March 2017. The last participant completed the study on the 30th October 2018.

Travel expenses were reimbursed but no other financial reimbursement was made to the participants for taking part.

3.1.2 Recruitment of Participants

Subjects were recruited from a database of volunteers that had consented to be contacted about research, held by the Memory Assessment & Research Centre (MARC), Southern Health NHS Foundation Trust; or via Join Dementia Research (JDR), an online self-registration service developed by the Department of Health and Alzheimer's Society which enables volunteers (both with and without memory impairment) to be matched to local dementia studies.

In order to be included in the study participants had to be over 50 years old, willing and able to give informed consent to participate and to be able to hear, read, write and perform neuropsychological tests in English. Healthy controls had to have no cognitive impairment evidenced by an SMMSE score > 27 and all AD subjects met NINCDS-ADRDA criteria for a diagnosis of Probable AD with an SMMSE score of 7-24 at screen.

Additionally, if any of the following exclusion criteria were met, subjects were not entered into the study:

1) Unlikely to cooperate in the study, not able to attend scheduled examinations and visits, or not able to follow study instructions.

2) Absence of a reliable study partner (AD subjects only)

3) Learning and linguistic/communication disabilities

4) History of eye disease (including glaucoma)

5) History of autoimmune disease

6) Current use of medications that have a diastolic effect on pupils

7) Current use of medications with a NA-based mechanism of action, such as selective noradrenaline reuptake inhibitors and α2-adrenoceptor antagonists

8) Current use of non-topical steroids or cytokine modulators

9) Vascular disorder (Modified Hachinski Ischaemic Scale score > 4)

10) History of major psychiatric disorder

11) History of alcohol or substance misuse within the last 2 years.

Potential participants who met inclusion/exclusion criteria during pre-screening were contacted by telephone, and if interested in participating, were sent the ethically approved participant information sheet. Information obtained from medical records held by Southern Health NHS and the participant's GP records, was reviewed to check for eligibility. If the participant volunteered to participate after considering the PIS, they were invited to attend MARC at Moorgreen Hospital to complete the consent process.

A pre-screening log listed all participants reviewed for the study with reasons as to why they weren't included, and an enrolment log was used to record of all consented participants.

3.1.3 Power calculations

As this study was designed as a pilot rather than aiming to demonstrate a definitive effect size, a sample size of 24 participants in each group was suggested by Clive Osmond from Statistical services. A total sample size between 24 and 50 is thought to be sufficient in most circumstances in pilot/feasibility studies in order to estimate variability in a continuous outcome measure with reasonable precision [303,304]. Based on Takahashi et al.'s study [275] which showed the LC contrast ratio to have a standard deviation of 3%, 24 participants in each group would give 80% power with 5% (two-sided significance) to determine a minimum significant decrease of 2.4% in LC contrast ratio. The lead physicist involved in this study, Dr Angela Darekar recommended recruiting an additional 4 healthy controls in order to optimise the MRI protocol. Therefore, a total of 24 AD participants and 28 healthy controls were recruited for this project.

3.1.4 Study Design

This was a cross-sectional observational study to investigate signal intensity changes within the LC detected using MRI, between AD and age and gender matched cognitively unimpaired subjects. All data was acquired over 3 visits, typically scheduled within a two-week period.

Visit 1 usually occurred at MARC, Moorgreen Hospital, however it sometimes occurred at the participant's home. After assessing for capacity, informed consent was obtained from all participants and their study partners prior to the start of any study-related procedures and in accordance with the Declaration of Helsinki and ICH Good Clinical Practice guidelines. Following this a full medical history was obtained including current medication, a history of any memory or other cognitive impairments, family history of AD, history of any MRI scans and completion of the MRI checklist (height and weight required) to ensure there were no contraindications to scanning. Psychometric and behavioural assessments were completed with the subject and study partner. Blood samples, an ECG and measures of autonomic function i.e., heart rate and blood pressure were obtained at multiple timepoints. The total visit time was 2 to 3 hours.

At Visit 2 all subjects underwent an hour-long scan using a Siemens 3-Tesla Skyra MR scanner and a 32-channel head coil at Southampton General Hospital.

Visit 3 took place in the eye-tracking lab in the Shackleton Building on Highfield Campus at the University of Southampton. Two experiments were conducted with each participant; a

pupillometry task to measure the pupil light reflex and a reading task to examine differences in eye movements during reading.

After completion of the eye tracking tasks, participants were debriefed about the study. Their GPs were informed of their participation and electronic secondary care records were updated where appropriate. All scales were second checked for scoring before data was inputted into SPSS.

3.1.4.1 Psychometric and behavioural assessments

The following well validated assessments were administered:

- Standardised Mini Mental State Examination (SMMSE) [305]. The SMMSE is an alternative, free version of the MMSE which is a well-established measure of cognitive function in elderly people to assess for dementia. It is widely used because it only takes 10-15 minutes to administer and has good test-retest and inter-rater reliability. There are 11 task items which assess orientation, memory recall, working memory, visuospatial skills and language. The maximum score is 30, with a score of 21 to 25 indicating mild dementia, a score of 13 to 20 indicating moderate dementia and a score under 12 indicating severe dementia.
- Montreal Cognitive Assessment (MoCA) [306]. The MoCA is a well validated and widely used tool used to assess cognitive impairment taking approximately 10 minutes to complete. It is more sensitive than the MMSE in being able to distinguish those with MCI from those that are cognitively unimpaired and those with mild dementia. It has 8 cognitive domains which include tests of visuospatial and executive function, memory, language, abstraction, attention, delayed recall and orientation. The maximum score is 30 with a score below 26 indicating impairment.
- Alzheimer's Disease Assessment Scale-cognitive sub-scale (ADAS-COG) [307]. This scale has 11 components which have been specifically designed to measure a wide range of cognitive deficits in AD. It is more comprehensive and more time-intensive than the MMSE, usually taking 45 minutes to 1 hour to complete, and is generally accepted as the standard measure of cognitive function for patients with mild to moderate AD in clinical trials.
- Pittsburgh Sleep Quality Index (PSQI) [308]. This is a 19-item scale completed with both the subject and study partner (for control participants it was only completed with the subject). It is a well-established measure of both sleep quality and quantity, widely used in psychiatric practice and research. A score greater than 5 indicates sleep disturbance.

- For the AD participants only, the Alzheimer's Disease Cooperative Study – Activities of Daily Living Inventory (ADCS-ADL) [309] was administered to the study partner. The ADCS-ADL consists of 23 questions answered by the informant about the amount of help they need to give the participant to complete various tasks. The score ranges from 0 to 78 with a lower score indicating greater functional impairment.
- For the AD participants only, the Neuropsychiatric Inventory (NPI) [310] was administered to the study partner to assess 12 behavioural disturbances that may feature in dementia: Delusions, Hallucinations, Agitation/Aggression, Depression/Dysphoria, Anxiety, Elation/Euphoria, Apathy/Indifference, Disinhibition, Irritability/Lability, Aberrant motor behaviour, Sleep and Night time Behaviour Disorders, Appetite and Eating Disorders. The informant rates the frequency and severity of the behavioural change and the distress this causes them as a carer.

Permission to use all assessments was sought prior to their use in this study. All assessments for this study were administered by me to eliminate any inter-rater bias. Examples of the scales are shown in Appendix C.

3.1.4.2 Physiological measures of autonomic function

Systolic and diastolic blood pressure in mmHg and heart rate in beats per minute (bpm) were obtained using an electronic sphygmomanometer under three conditions which all aimed to elicit a response from the sympathetic nervous system in order to give an indirect measure of LC function. For each of the following tasks, blood pressure and heart rate were recorded both before and after the task and the difference between the two readings was assessed for change.

Orthostatic reflex – Participants were laid in a supine position and blood pressure and heart rate was obtained. Participants were asked to stand, and these measures were immediately taken again and then after the subject had been standing for 3 minutes they were repeated for a final time. When the participant stands abruptly a large volume of blood in excess of 500ml [311] will gather in their legs which is reflected by a fall in systolic and diastolic blood pressure as well as an increase in heart rate. This should return to normal within 1-2 minutes. However if the reductions are sustained after 3 minutes measured as a greater than 20mmHg fall in systolic BP and more than a 10mmHg fall in diastolic BP, this indicates an abnormal response termed orthostatic hypotension [311].

Serial Subtraction – Subjects were asked to start with the number 100 and minus 7 and then keep subtracting 7 from each subsequent answer for 60 seconds. Some participants started at 700 to

ensure the task was sufficiently challenging. Systolic blood pressure to this psychological stressor should increase by at least 10mmHg [312,313].

Cold pressor test – Subjects immersed their left hand in ice cold water for 90 seconds. The pain and emotional arousal associated with the task should activate the sympathetic nervous system and lead to an increase in both heart rate and blood pressure due to peripheral vasoconstriction. An average increase of 20mmHg in systolic blood pressure, 15mmHg in diastolic blood pressure and of heart rate by 10 bpm is typically observed, indicating a working LC-NA system [312,314]

3.1.4.3 Blood Samples

A sample of 10ml venous blood was obtained for each participant at approximately the same time of day (between 10am and 12pm) to minimise the effect of circadian rhythms on the data.

The sample was centrifuged for 15 minutes at 2500rpm, and the cell free-serum was pipetted into 4 aliquots which were stored at -80°C and were only identifiable by the participant study number. All samples were analysed together at one time to examine serum levels of cytokines including TNF-α, IL-6, and IL-10 which were measured using a multiplex immunoassay - Meso Scale Discovery (MSD, V-PLEX Pro-inflammatory Panel) in a Clinical and Experimental Sciences laboratory at Southampton General Hospital by Dr Laurie Lau. In addition, he also measured the serum APOE ε4 using the RayBio Human ApoE4 ELISA Kit.

3.1.4.4 MRI Acquisition

All images were obtained on the same scanner, a Siemens 3-Tesla Skyra MR scanner and a 32-channel head coil. The neuromelanin-sensitive MRI protocol is a T1 weighted turbo spin echo sequence with TR=600ms, TE = 16ms, flip angle = 90°, 5 signal averages, 220mm FOV, pixel size = 0.4mm x 0.4mm, slice thickness 2.5mm, 12 slices, acquisition time 10 minutes 21 seconds.

For the acquisition of the LC images, sat bands were placed perpendicular to the fourth ventricle ensuring that the inferior colliculi and the base of the fourth ventricle were included as in Figure 7 below.

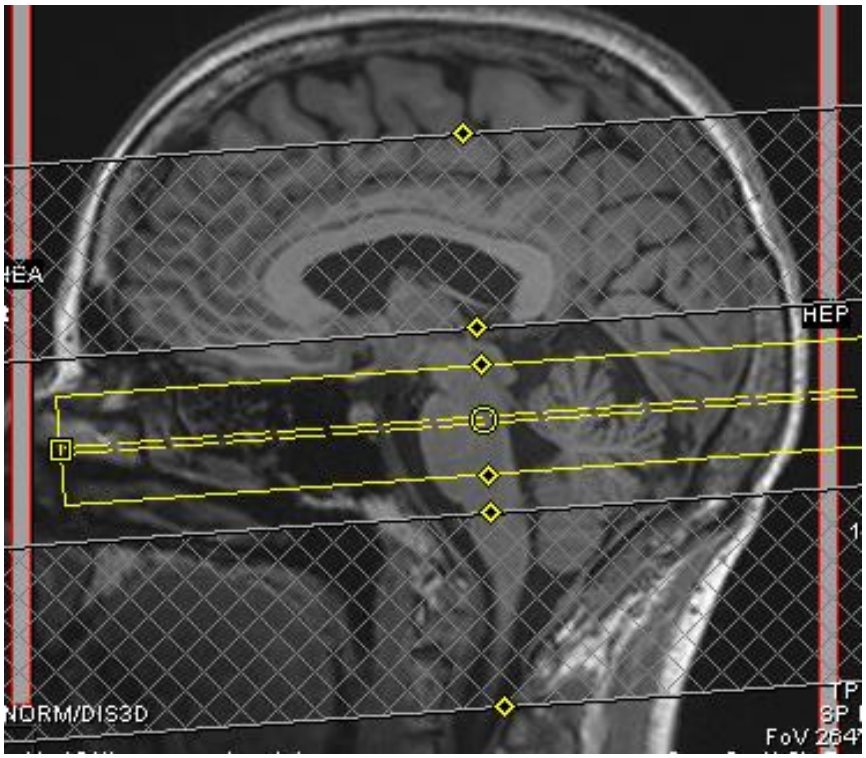


Figure 7. Placement of the sat bands for the LC NM-MR imaging protocol.

This protocol allows the LC to be visualised often by eye on axial images as two small hyperintense areas, sometimes seen over multiple axial slices, see Figure 8.

Standard clinical imaging protocols were obtained for structural analysis and to check for abnormalities including a sagittal Alzheimer's Disease Neuroimaging Initiative (ADNI) sequence. Additionally other exploratory images were obtained including susceptibility weighted imaging (SWI) to examine any link between iron deposition and LC degeneration and resting state fMRI to look at the functional connectivity of the LC with other brain areas at rest to explore how these may be disrupted in people with AD.

The sequences used in this study were chosen by Dr Angela Darekar. The MR images were obtained by radiologists at Southampton General Hospital under the supervision of Chris Everitt and Chris Watson and were provided to me on an anonymised disc so that I could perform the analysis of the images blind to subject group.

3.1.4.5 MRI Image Analysis

The LC can be seen on some images at the opening of the 4th ventricle as two hyperintense areas as shown in Figure 8.

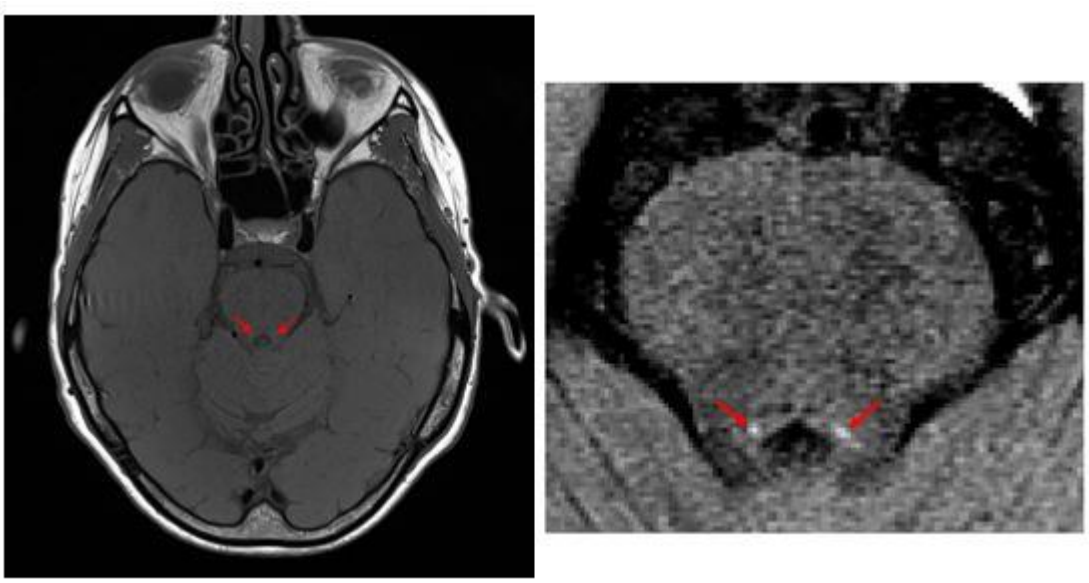


Figure 8. NM-MR image of the LC denoted as two hyperintense areas (red arrows). The axial slice depicted here is level 3 of the 4 consecutive slices measured.

Due to its length, the LC is often visible on multiple axial slices on MRI. To best capture changes in the rostro-caudal extent of the LC signal intensity (SI), SI was measured across 4 consecutive slices. It has been hypothesised that the LC is topically organised and that LC cell loss in AD may be more selective for the rostral and middle sections of the LC than the caudal aspect and so by examining the signal over several slices we hoped to test this theory too.

The SN and cerebral aqueduct (CA) were usually visible on the images. The last slice which the SN, CA & inferior colliculi (IC) can be seen was considered to be level 1 and the contrast ratio of the LC (LC-CR) in this slice and the three consecutive slices was calculated using ImageJ by myself as a blinded rater. An example of these consecutive levels is shown in Figure 9.

As per previous studies [275] it was expected that the LC signal would be greatest at level 3 which is the axial slice that corresponds to approximately 7mm below the IC (two slices). However, the analysis will also be performed using the axial slice where the LC signal is brightest for each individual and finally, the LC signal will be averaged across all 4 slices to see whether differences can be observed when looking at the whole LC to see which gives best results.

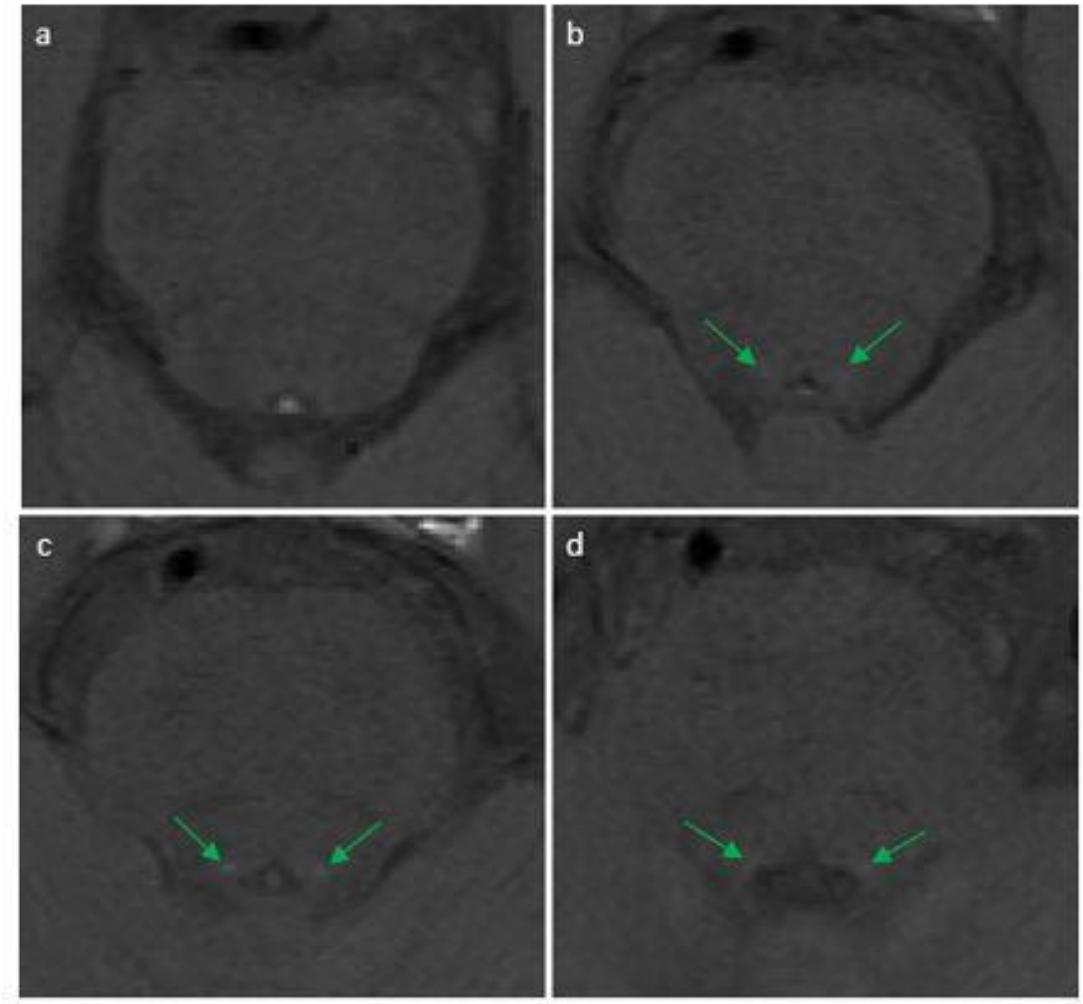


Figure 9. Consecutive axial slices through the pons, Level 1 (a) to 4 (d).

a) Level 1 where the cerebral aqueduct opens up into the fourth ventricle to b) Level 2, c) Level 3 in which the LC signal is brightest (green arrows) and d) Level 4 in which the 4th ventricle has opened out fully.

Similar methods as those used previously were employed to calculate the LC-CR in this study [275,276,284,285]. In Image J, for each axial image, the pons was segmented using the freehand selection tool and cropped to create a mask which was then smoothed. The mean signal intensity of a circular ROI of 99.701mm² in the pons tegmentum was calculated known as SIPT and was used as a reference region to compare the LC signal to.

The location of the LC was determined by using the find maxima function and adjusting the noise tolerance until the two brightest points remained in the area anatomically accurate for the LC on both sides of the brainstem, based on previous post-mortem and NM-MRI studies. A circular ROI of 1.67mm² was centred on this brightest pixel on both the left and right side and the mean signal intensity was calculated for each side. These were then averaged together to get the overall LC signal intensity, SILC. This was then compared to the reference region to provide a contrast ratio

for each side of the LC (left & right), calculated using the formula LC-CR = (SILC – SIPT)/SIPT, as shown in Figure 10.

The analysis was repeated on 3 separate occasions a minimum of a week apart to demonstrate good reliability of this method.

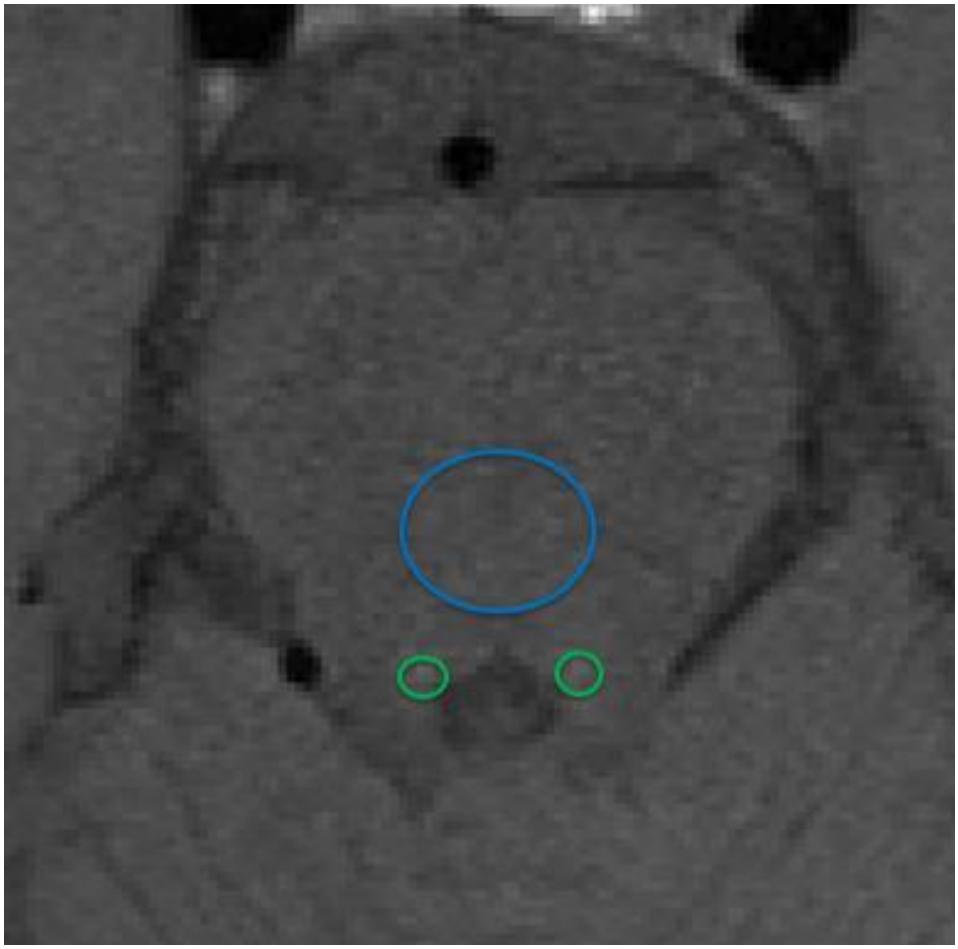


Figure 10. MR image to illustrate how the LC-contrast ratio was calculated

LCSI calculated from the average SI of the mean RLC ROI and mean LLC ROI minus the mean signal of the pons tegmentum divided by the signal intensity of the pons tegmentum (LCSI – SIPT)/SIPT

3.1.4.6 Pupillometry

For the pupil light response task, an eye tracker (EyeLink 1000+) was used to continuously record pupil size at one sample per second. Subjects were required to fixate on a screen displaying a white cross on a black background with the lights off to get a baseline pupil size measurement. After 25 seconds there was a light flash displayed for 200 milliseconds followed by a further 25 seconds of the fixation cross on the same black background as the baseline. The light flashes had 4 luminance levels (Level 1 being the brightest and Level 4 being the least luminant), each seen 4

times so that each participant completed 16 trials. The order of these trials was randomised for each participant. 16 trials was thought to be sufficient as previous studies have successfully used 12 trials [171]. The light flash only occurred after 25 seconds if the participant did not move their eyes and remained fixated on the cross so as to not cause pupil foreshortening. A schematic for the experiment is shown below, Figure 11.

The task was explained to the subject and instructions were repeated throughout as necessary. The eye tracker was calibrated to each participant using 3 points on a central horizontal line and calibration was performed every 4 trials at which time subjects were given a break from looking at the screen if needed.

This experiment was designed by Dr Gemma Fitzsimmons. I administered this experiment to all of the participants.

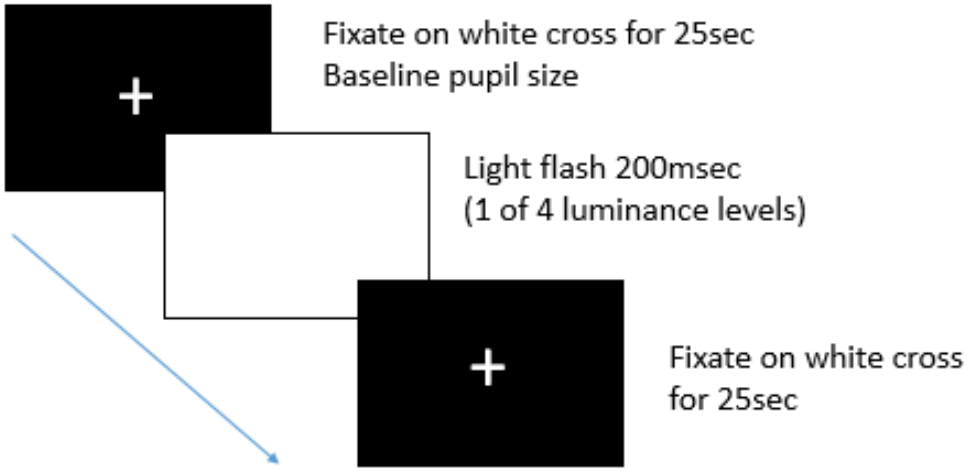


Figure 11. Schematic to show an example trial from the pupil light reflex experiment.

Baseline pupil size is calculated during the 25 seconds before the light flash. The maximum constriction size of the pupil caused by each flash as well as the time taken in msec to reach maximum constriction is also recorded.

3.1.4.7 Pupil Light Reflex Analysis

Data from the pupil light reflex experiment was analysed by Dr Gemma Fitzsimmons who was able to provide for each participant and for each light level for the 16 trials:

- the mean baseline pupil size recorded in the 25 seconds prior to the light flash (in mm)

- the amplitude of the light reflex response i.e. the difference between the baseline pupil size preceding the light flash and the maximum constriction size of the pupil during the light flash (in mm)
- the time taken to reach the maximum constriction during the light flash (in msec)

3.1.5 Statistical Analysis

Statistical analysis on the data was performed using the software IBM SPSS v24 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

Normality of the data was verified for each variable using histograms, descriptive statistics, Q-Q plots and Shapiro-Wilk test. Where data was normally distributed, independent samples t-tests and analysis of Variance (ANOVA) tests were used to compare differences between the participant groups. Where data was not normally distributed the Kruskal-Wallis test was used to compare the data across groups. When a significant group effect was found, pairwise comparisons were made to determine which groups differed significantly from each other. Pearson's or Spearman's rank correlations were used as appropriate to explore the relationship between the signal intensity of the LC expressed as a contrast ratio with other clinical measures and indirect measures of LC activity. A threshold of 5% was used for intergroup comparisons and Bonferroni corrected *p* values were used for correlations to account for multiple comparisons. Linear regression analysis was performed to determine to what extent differences in LC signal on the MRI were due to differences in age, gender or diagnosis using LC-CR as the outcome variable and age, gender and group (control and AD) as input variables.

3.2 Results

3.2.1 Participants

Initially four control subjects with no cognitive impairment were recruited to test the imaging protocol. Once the imaging protocol was determined an additional 48 subjects (28 male, 20 female) were recruited to this study. Of these, 24 were subjects with a diagnosis of Alzheimer's Disease (14 male, 10 female age 58 – 82) and 24 were age and gender matched control subjects with no cognitive impairment (14 male, 10 female, age 57 – 82). An additional 7 participants were consented but were unable to have the MRI scan and were considered to be screen failures (2 due to contra-indications to MRI scanning, 4 who could not tolerate the MRI procedure and 1 that was lost to follow up).

Two participants with AD had to be excluded from the analysis due to poor quality scans, which made identifying the LC unclear and so their age and gender matched controls were also excluded from the analysis for this study:

- LC07 – Accurate LC-contrast ratios could not be calculated for this subject due to blurring of the image of the LC with the cerebral aqueduct evident as a large hyperintensity in the location of the LC and fourth ventricle. When an attempt to calculate a LC-CR value using the same method was made, the result was much higher than for every other participant and so is unlikely to be accurate.
- LC11 – This subject was the age and gender matched control of LC07 and so was also removed from the analysis.
- LC58 – The quality of the scan was poor with motion artefact and so the LC-CR could not be accurately calculated.
- LC03 – This subject was the age and gender matched control for LC58 and so was also removed from the analysis.

Therefore, in the final analysis there were a total of 22 control subjects, 11 subjects with mild AD (scoring between 22 and 25 on the SMMSE) and 11 subjects with moderate AD (scoring between 7 and 20 on the SMMSE). Baseline characteristics of the three groups of participants are shown in Table 3.

Table 3. Characteristics i.e., MRI, Gender, BMI and SMMSE score for each participant group

	Age at MRI mean ±SD (range)	**Gender**	**SMMSE score median [IQR]**	**BMI mean ±SD**
Control (*n*=22)	72.5 ±5.9 (57 to 82)	13M, 9F	30 [29-30]	26.25±3.4
Mild AD (*n*=11)	71.3 ±5.6 (58 to 78)	5M, 6F	24 [24-24]	25.9 ±3.6
Moderate AD (*n*=11)	73.9 ±6.1 (57 to 82)	8M, 3F	17 [16-20]	28.09 ±4.3

Results are presented as mean ± standard deviation for normally distributed data or median [interquartile range] for non-parametric data. *M* male, *F* Female

Data for age were normally distributed. An ANOVA found no significant difference in age at MRI between the 3 groups $F(2,41) = 0.533$, $p=0.591$ (this is to be expected as controls were age matched to AD participants).

There was no significant difference in the proportion of males to females across the 3 groups as determined by Pearson Chi-Squared test, $\chi^2(2) = 1.692$, $p=0.429$.

Data for BMI were normally distributed. An ANOVA showed no significant difference in BMI between the 3 groups $F(2,40) = 1.165$, $p=0.322$.

3.2.2 Psychometric and behavioural assessments

SMMSE data were not normally distributed. A Kruskal-Wallis H test showed a statistically significant difference in SMMSE total score between the different participant groups, $H(2) = 37.931$, $p<0.001$, with a mean rank score of 33.5 for the control group, 17 for the mild AD group and 6 for the moderate AD group. Post-hoc comparisons revealed that controls scored significantly higher than both AD groups ($p<0.001$), however there was no significant difference between those with mild AD and those with moderate AD ($p=0.12$).

ADAS-Cog total scores were normally distributed, and Analysis of Variance (ANOVA) showed groups to differ significantly on total score, $F(2,41) = 51.743$, $p<0.001$. Post hoc comparisons using the Tukey HSD test indicated that the moderate AD group scored significantly higher (m=29.21, SD=11.58, $p<0.001$) than the mild AD group who in turn scored significantly higher (m=16.47, SD=4.23, $p<0.001$) than the control group (m=5.44, SD=2.7, $p<0.001$). ADAS-Cog total scores are inverted so that a higher score corresponds to greater cognitive impairment with the moderate AD group therefore showing greater cognitive impairment.

MoCA scores were not normally distributed within the control group (Shapiro Wilk, $p=0.046$). A Kruskal-Wallis H test showed a statistically significant difference in MoCA total score between the different participant groups, $H(2) = 26.302$, $p<0.001$, with a mean rank score of 25 for the control group, 10 for the mild AD group and 3 for the moderate AD group. Post-hoc comparisons revealed that controls scored significantly higher than both AD groups ($p<0.001$), however there was no significant difference between those with mild AD and those with moderate AD ($p=0.218$).

ADCS-ADL and NPI scores were only obtained from the AD participant groups who enrolled with a study partner. Control participants were considered to be independent in activities of daily living. Data for ADL scores were not normally distributed. Moderate AD participants (mean rank=8.14) were significantly more impaired in activities of daily living than mild AD group (mean rank=14.86) as determined by the Mann Whitney U test ($U = 23.5$, $p=0.015$). NPI scores were not normally distributed. A Mann Whitney U test revealed no difference in total score on the NPI between mild AD (mean rank=11.73) and moderate AD groups (mean rank=11.27), $U = 58$, $p=0.898$, indicating both groups had similar levels of behavioural impairments noted by their study partners.

PSQI scores were not normally distributed. Kruskal-Wallis test was not significant indicating that the distribution of the PSQI total scores was the same across the three groups, $H(2) = 3.2$, $p=0.202$.

A summary of the scores from all the psychometric and behavioural assessments by participant group is shown in Table 4.

Table 4. Summary of cognitive and behavioural test scores for the control, mild AD and moderate AD groups

	Control (*n*= 22)	**Mild AD (*n*=11)**	**Moderate AD (*n*=11)**	***p* value**
SMMSE	30 [29-30]	24 [24-24]	17 [16-20]	*p*<0.001
ADAS-Cog	5.44 ±2.70	16.49 ±4.23	29.21 ±11.58	*p*<0.001
MoCA	27 [26-29] (*n*=21)	19 [18.5-21.5] (*n*= 9)	11 [10-15] (*n*=5)	*p*<0.001
ADCS-ADL	n/a	60 ± 17	69 ± 6	*p*=0.015
NPI	n/a	8 [1-16]	5 [4-12]	*p*=0.671
PSQI	4.5 [3-7.25]	3 [2-4]	2 [1-5]	*p*=0.202

*$p<0.05$

p values calculated using either ANOVA or Kruskal-Wallis test

Data presented as either mean ±standard deviation for normally distributed data or median [Interquartile range] for non-parametric data

3.2.3 Physiological measures of autonomic function

Data for blood pressure measurements were normally distributed.

Orthostatic reflex – A paired samples t test found there to be a 6.8mmHg decrease in systolic BP on standing compared to supine BP in the control group, $t(21) = 2.468$, $p=0.022$, but no change in the AD group, $t(17) = 0.279$, $p=0.783$. Independent samples t test found no difference between AD and control participants, $t(39) = -1.579$, $p=0.123$. There was no change in diastolic BP between these two conditions for either the control group, $t(21) = 0.142$, $p=0.888$ or the AD group, $t(17) = -0.095$, $p=0.926$. Both control and AD groups had an increase in heart rate. In controls the mean increase was 8bpm, $t(21) = -5.561$, $p<0.001$ and in AD patients the mean increase was 6bpm, $t(17) = -4.892$, $p<0.001$. There was no difference in heart rate increase between those with AD and controls, $t(39) = 1.088$, $p=0.283$.

After 3 minutes of standing, systolic BP was no longer different from supine measurements in control participants, $t(17) = 2.012$, $p=0.057$ and remained indifferent for AD participants, $t(17) = 0.378$, $p=0.756$. Heart rate also remained increased in control participants with a mean increase of 7bpm, $t(21) = -5.084$, $p<0.001$ and in AD participants with a mean increase of 6bpm, $t(17) = -4.030$, $p<0.001$.

Serial Subtraction - There were no differences in systolic or diastolic BP or HR from rest following the serial subtraction task for either the control or AD groups.

Cold pressor test – A paired samples t test found there was a significant increase in systolic BP from rest following the cold pressor task for control participants, mean increase 6.5mmHg from rest $t(19) = -2.108$, $p=0.049$, but not for AD participants, $t(11) = -2.030$, $p=0.067$. Similarly there was a significant increase in diastolic BP from rest following the cold pressor test in the control group, mean increase = 7.6mmHg, $t(19) = -2.226$, $p=0.038$, but not for the AD group, $t(11) = -0.661$, $p=0.522$. There was no difference in heart rate between the two conditions for either the control group, $t(19) = -0.283$, $p=0.780$ or the AD group, $t(11) = -0.931$, $p=0.372$.

3.2.4 LC Signal intensity differences

3.2.4.1 LC-CR differences at each axial slice

LLC and RLC contrast-ratios were averaged together for each participant and were compared between the control and AD groups over 4 consecutive axial slices. Level 1 corresponds to the slice that shows the opening of the cerebral aqueduct and Level 4 ends at the point where the 4th ventricle has widened fully. Significant differences were further examined by comparing controls, mild AD and moderate AD groups to see how early on in the disease process they occur.

Level 1 – Data were normally distributed for all three groups on this axial image. An Independent samples t-test found no significant differences in LC-CR between the control and AD groups, $t(40) = 1.625$, $p=0.112$ and ANOVA found no difference in LC-CR between the control, mild AD or moderate AD groups, $F(2,39) = 1.443$, $p=0.248$.

Level 2 – Data were normally distributed for all three groups on this axial level. An Independent samples t-test found no significant differences in LC-CR between the control and AD groups, $t(42) = 1.678$, $p=0.101$ and ANOVA found no difference in LC-CR between the control, mild AD or moderate AD groups, $F(2,41) = 1.401$, $p=0.258$.

Level 3 – Data were normally distributed. An independent samples t-test comparing control and AD groups was significant, $t(42) = 3.228$, $p=0.002$. ANOVA found a significant difference in the average LC-CR between the groups, $F(2,41) = 5.095$, $p=0.011$. Post-hoc comparisons revealed that the mean LC-CR in the control group (m=0.16, SD=0.04) was significantly greater than the LC-CR of the mild AD group (m= 0.12, SD=0.04, $p=0.039$) and the moderate AD group (m=0.12, SD=0.04, $p=0.028$). However, there was no significant difference between the mild AD group and the moderate AD group ($p=0.992$).

Level 4 – Data were normally distributed for all three groups. An Independent samples t-test found no significant differences in LC-CR between the control and AD groups, $t(40) = 1.921$,

p=0.062 and ANOVA found no difference in LC-CR between the control, mild AD or moderate AD groups, $F(2,39) = 2.303$, *p*=0.113.

Mean LC-CRs for each group at each axial level are shown in Table 5.

Table 5. Mean LC-CRs for each axial level in control, mild AD and moderate AD groups.

	Level 1 mean ±SD	**Level 2 mean ±SD**	**Level 3 mean ±SD**	**Level 4 mean ±SD**
Control (n=22)	0.11 ±0.03	0.13 ±0.03	**0.16 ±0.03**	0.12 ±0.04
Mild AD (n=11)	0.09 ±0.03	0.11 ±0.03	**0.12 ±0.04**	0.10 ±0.03
Moderate AD (n=11)	0.10 ±0.02	0.11 ±0.05	**0.12 ±0.04**	0.09 ±0.04

***p<0.01**

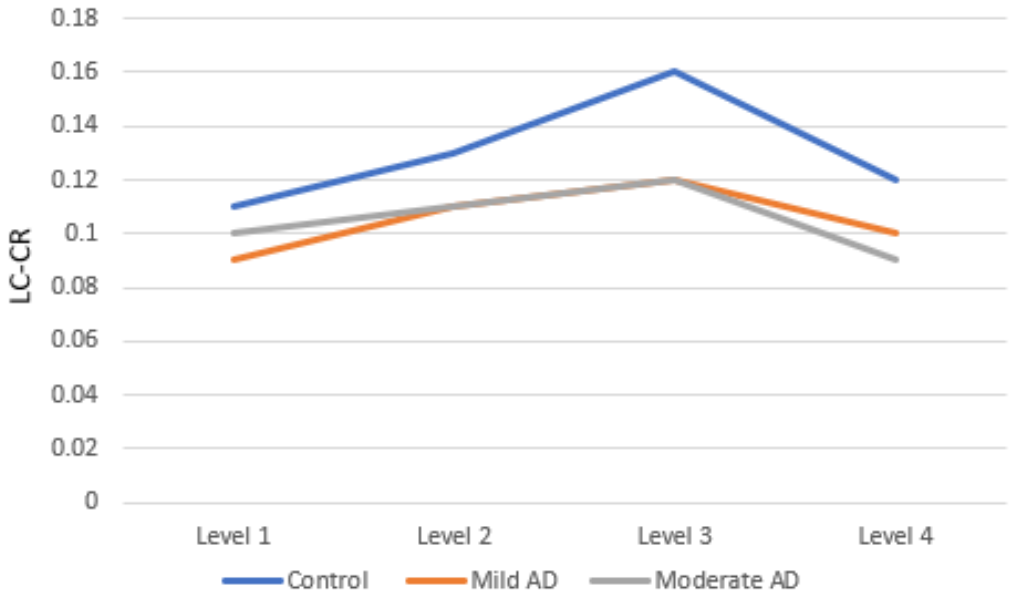


Figure 12. A graph to show LC-CR differences across 4 consecutive axial sections in subjects with mild and moderate AD compared to age and gender matched controls

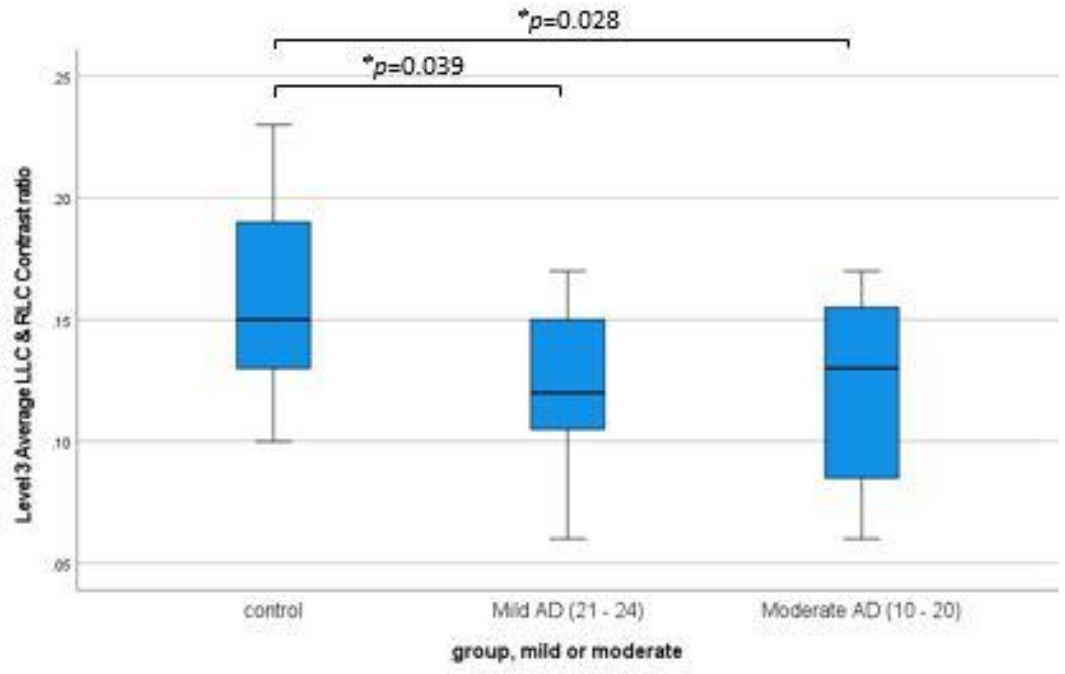


Figure 13. At Level 3, mean LC-CRs in the control group were significantly greater than in both the mild and moderate AD groups.

3.2.4.2 LC-CR average across all 4 slices

An average across all 4 levels was calculated to look at the average contrast ratio of the LC in its entirety. Data for the average LC-CR for the entire LC were normally distributed. Independent samples t test comparing control and AD groups was significant, $t(42) = 3.126$, $p=0.003$. An ANOVA found a significant difference in the average LC-CR between the groups, $F(2,41) = 5.371$, $p=0.008$. Post-hoc comparisons revealed that the mean LC-CR in the control group (m=0.13, SD=0.03) was significantly greater than the LC-CR of the moderate AD group (m=0.10, SD=0.03, $p=0.009$), shown in Figure 14. However, there was no difference between the control group and the mild AD group (m=0.11, SD=0.03, $p=0.13$) or between the mild AD and moderate AD groups ($p=0.588$).

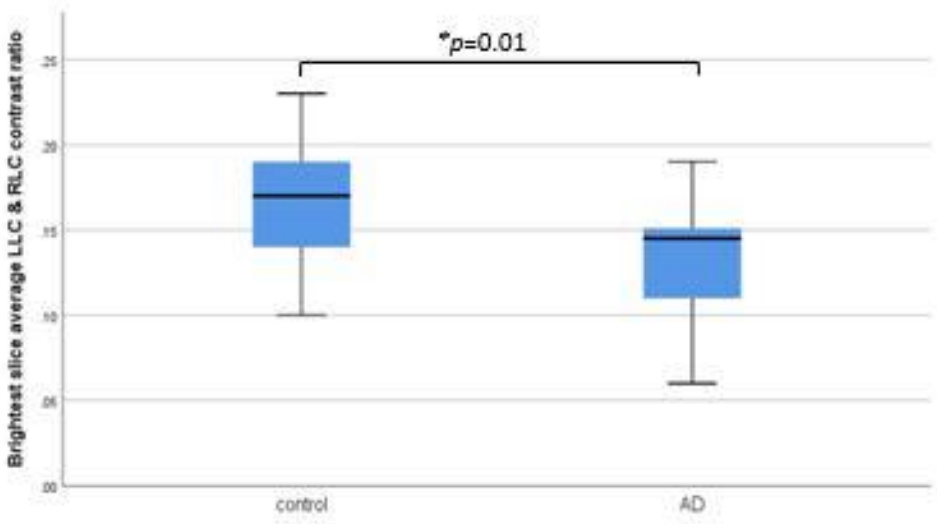


Figure 14. The average LC-CR for the whole LC is significantly lower in those with moderate AD compared to controls.

Lines represent median values

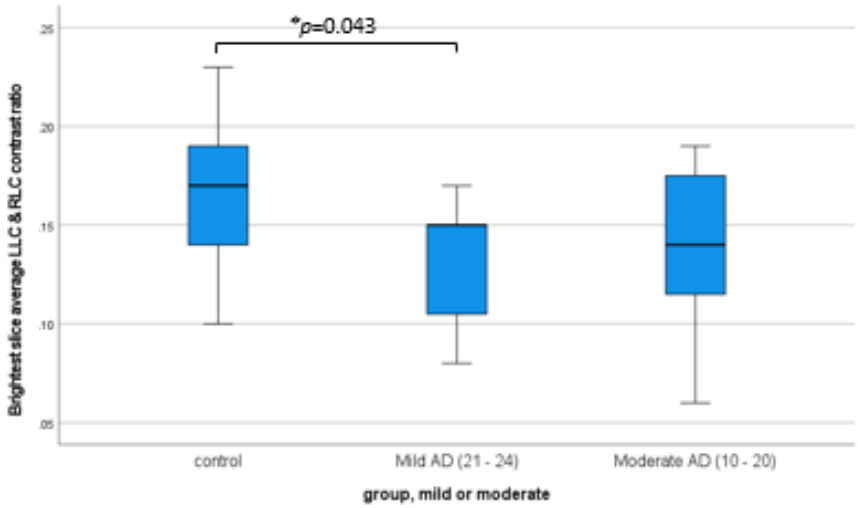


Figure 15. Control participants had a significantly greater LC-CR compared to those with Mild AD

3.2.4.3 LC-CR differences where signal is strongest

As there is individual variation in both the length of the brainstem and the length of the LC, for some participants the third consecutive slice sampled (Level 3) was much lower down the brainstem compared to others. Therefore, for each participant the axial image in which the LC was most visible (termed the brightest slice) was used in the analysis to look for differences

between the three groups. For most participants this was still Level 3 but for others it was Level 2 or Level 4.

Data for the LC-CRs on this brightest slice were normally distributed. Independent samples t test comparing control and AD groups was significant, $t(42) = 2.695$, $p=0.01$. A one-way ANOVA comparing means for the three groups was significant $F(2,41) = 3.719$, $p=0.033$. The mild AD group had a significantly lower LC-CR (m=0.13, SD=0.03) than controls (m=0.16, SD=0.03), $p=0.043$. Although the moderate AD group (m=0.14, SD=0.04) also had a numerically lower LC-CR than controls, this difference did not meet reach significance, $p=0.161$. There was no difference in LC-CR between mild AD and moderate AD groups, $p=0.850$.

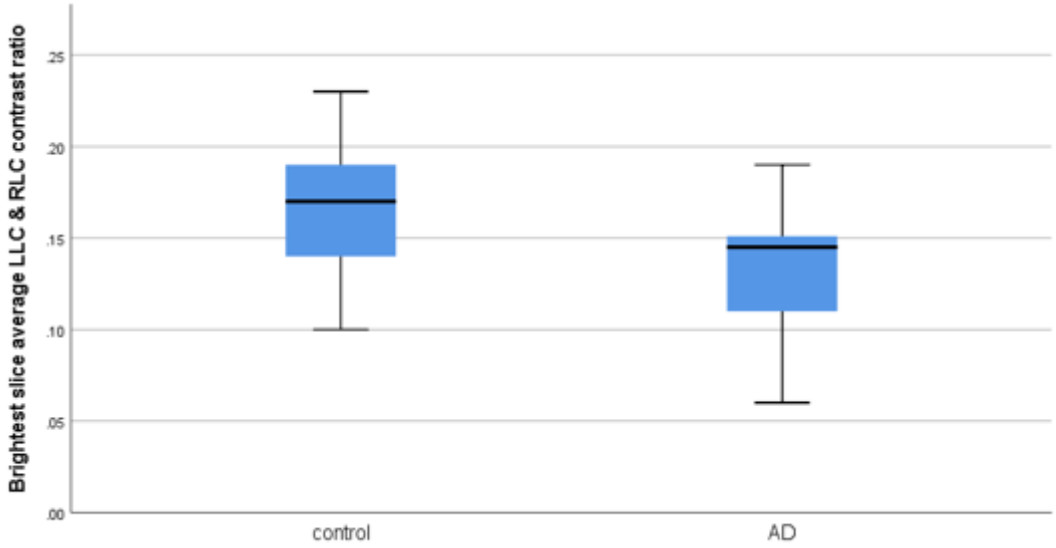


Figure 16. Mean LC-CRs in the control group were significantly greater than the AD group ($p=0.01$) when sampling from the image where the signal is brightest

Although all methods for quantifying the LC-CR signal gave similar results, using the axial slice where the signal was strongest had the best face validity.

3.2.5 Signal intensity differences between left and right LC

Using the level where the signal of the LC was greatest for each participant (brightest slice), the data for the LLC-CR and RLC-CR were normally distributed for both AD and control groups. A within-subjects t-test indicated that the RLC had a significantly greater CR (m=0.17, SD=0.04) than the LLC (m=0.13, SD=0.04), $t(43) = -8.507$, $p<0.001$. This was not related to handedness.

Independent samples t-tests showed the AD group had a significantly lower CR on both the left (m=0.14, SD=0.04), $t(42) = 2.209$, $p=0.033$, and right side of the LC (m=0.15, SD=0.04), $t(42) = 2.77$, $p=0.008$, when compared to controls (left LC m=0.17, SD=0.04, right m=0.19, SD=0.04).

A one-way ANOVA found a significant difference in LC-CR on the right side between the three groups, $F(2,41) = 3.982$, $p=0.026$ and Tukey test found controls to have a greater RLC-CR compared to mild AD, $p=0.033$, but no difference between controls and moderate AD or mild and moderate AD. However, ANOVA found no differences between groups for the left LC-CR, $F(2,41) = 2.579$, $p=0.088$.

Table 6. The RLC had a significantly brighter contrast ratio than the LLC for both AD and control groups ($p<0.001$).

The LC-CRs were lower in the AD group on both sides when compared to controls, (LLC $p=0.033$, RLC $p=0.008$).

	RLC LC-CR	**LLC LC-CR**	***p* value**
Control ($n=22$)	0.19 ±0.04	0.14 ±0.04	$p<0.001$
AD ($n=22$)	0.15 ±0.04	0.12±0.04	$p<0.001$
p value	$p=0.008$	$p=0.033$	

Data presented as mean ±standard deviation

3.2.6 Effect of Age on LC signal intensity

Data for age were normally distributed. A Pearson product-moment correlation coefficient was computed to assess the relationship between age and LC-CR when looking at the brightest slice. There was no correlation between the two variables for controls (r=0.166, $n=22$, $p=0.461$) or for AD participants (r= -0.227, $n= 22$, $p=0.31$).

3.2.7 Gender differences

LC signal data were normally distributed for both men and women. In the control group, females had a significantly lower mean LC-CR (m=0.14, SD=0.03) than males (m=0.18, SD=0.02) as shown by an independent samples t-test, $t(20) = 4.08$, $p=0.001$. In the AD group, females also had a significantly lower average LC-CR (m=0.12, SD=0.03) than males (m=0.15, SD=0.04) as shown by an independent samples t-test, $t(20) = 2.277$, $p=0.034$.

As women (mean rank 17.36) were significantly younger than men (mean rank 26.06), Mann Whitney U test ($U = 141.5$, $p=0.027$), linear regression analysis was performed to determine whether age influenced the relationship between the LC-CR and gender (using LC-CR as the dependent variable), but gender remained significant, $p=0.001$, adjusted mean difference = 0.044.

Linear regression analysis to see if the significant difference in LC-CR was affected by age and gender using LC-CR as the outcome variable and age, gender and group (control and AD) as input variables found the relationship between group and LC-CR remained, p=0.002, mean difference -0.28, confidence interval -0.011 - -0.045; age remained insignificant, p=0.078, mean difference -0.001, confidence interval -0.00 - -0.003 and gender remained insignificant, p<0.001, mean difference -0.043, confidence interval -0.025 - -0.062

3.2.8 Correlating LC-CRs with cognitive tests

SMMSE score was positively associated with LC-CR, r_s = 0.325, p=0.032. There were no significant correlations between LC-CR and the other cognitive test scores i.e., MoCA r_s = 0.148, p=0.397, ADAS r = -0.270, p=0.076, NPI r_s = -0.257, p=0.247 and ADL r_s = 0.041, p=0.855. Once a Bonferroni corrected significant p value of 0.01 was applied, the association between SMMSE score and LC-CR was no longer significant.

There was no significant association between LC-CR and PSQI total score r_s = 0.146, p=0.344.

3.2.9 Pupil light reflex

Data for baseline pupil size, mean difference in pupil size following the light flash and the time taken for the pupil to reach maximum constriction were normally distributed for all four light luminance levels.

Luminance level 1 - no differences were observed between the control and AD group in the baseline pupil size t(40) = 1.811, p=0.078; the time taken for the pupil to reach maximum constriction t(40) = 1.583, p=0.121, and the mean difference in pupil size t(40) = 1.837, p=0.074 following the light flash. There were also no differences in these measures when comparing control, mild AD and moderate AD groups, F(2,39) = 1.724, p=0.192, F(2,39) = 2.423, p=0.102 and F(2,39) = 1.892, p=0.164 respectively.

Luminance level 2 - control participants had a significantly larger baseline pupil size (m=5.07, SD=1.23) than the AD participants (m=4.37, SD=0.92), t(40) = 2.109, p=0.041. However this difference was not significant when comparing control, mild AD and moderate AD groups F(2,39) = 2.307, p=0.113. Control participants also had a greater difference from baseline pupil size to maximum constriction size (m=0.67, SD=0.16) than AD participants (m=0.59, SD=0.13), t(40) = 2.139, p=0.039. However again this difference was not significant when comparing control, mild AD and moderate AD groups F(2,39) = 2.403, p=0.104. There was no difference in time taken for the pupil to reach maximum constriction after the light flash when comparing control and AD

groups $t(40) = 0.680$, $p=0.50$ or when comparing control, mild AD and moderate AD groups $F(2,39) = 2.979$, $p=0.063$.

Luminance level 3 - no differences were observed between the control and AD group in the baseline pupil size; $t(40) = 1.743$, $p=0.089$, the time taken for the pupil to reach maximum constriction, $t(40) = 1.458$, $p=0.153$, and the mean difference in pupil size at the light flash $t(40) = 1.127$, $p=0.266$. There were also no differences in these measures when comparing control, mild AD and moderate AD groups, $F(2,39) = 1.655$, $p=0.204$, $F(2,39) = 1.657$, $p=0.204$ and $F(2,39) = 1.162$, $p=0.323$ respectively.

Luminance level 4 - no differences were observed between the control and AD group in the baseline pupil size, $t(39) = 1.425$, $p=0.162$; the time taken for the pupil to reach maximum constriction, $t(39) = 0.942$, $p=0.352$; and the mean difference in pupil size at the light flash $t(39) = 0.534$, $p=0.596$. There were also no differences in these measures when comparing control, mild AD and moderate AD groups, $F(2,38) = 0.991$, $p=0.381$, $F(2,38) = 0.593$, $p=0.557$ and $F(2,38) = 0.232$, $p=0.794$ respectively.

Means for each pupil measure for each luminance level for each group along with the test statistics are displayed below in Table 7.

Table 7. Table to show pupillometry measures for AD and control participants across 4 luminance levels.

Measurements include average baseline pupil size (mm), mean difference in the amplitude of the pupil constriction (mm) and the time taken to reach the constriction (msec). Means ± standard deviations, test statistic used and significance value displayed.

	Control (n=21)	AD (n=21)	test statistic and *p* value
Luminance level 1			
Baseline pupil	5.04 ±1.28	4.41 ±0.95	$t(40) = 1.811$, $p=0.078$
Amplitude	0.68 ±0.17	0.60 ±0.13	$t(40) = 1.837$, $p=0.074$
Constriction velocity	435.05 ±62.93	464.56 ±57.93	$t(40) = 1.583$, $p=0.121$
Luminance level 2			
Baseline pupil	**5.07 ±1.23**	**4.367 ±0.92**	**$t(40) = 2.109$, $p=0.041$**
Amplitude	**0.69 ±0.16**	**0.59 ±0.13**	**$t(40) = 2.139$, $p=0.039$**
Constriction velocity	428.6 ±58.05	440.47 ±55.06	$t(40) = 0.680$, $p=0.50$
Luminance level 3			
Baseline Pupil	5.00 ±1.23	4.44 ±0.84	$t(40) = 1.743$, $p=0.089$
Amplitude	0.68 ±0.17	0.62 ±0.18	$t(40) = 1.127$, $p=0.266$
Constriction velocity	433.18 ±72.55	402.16 ±65.11	$t(40) = 1.458$, $p=0.153$
Luminance level 4			
Baseline pupil	4.99 ±1.25	4.51 ±0.87	$t(39) = 1.425$, $p=0.162$
Amplitude	0.68 ±0.17	0.65 ±0.19	$t(39) = 0.534$, $p=0.596$
Constriction velocity	388.44 ±72.02	409.43 ±70.57	$t(39) = 0.942$, $p=0.352$

Repeated measures ANOVAs were used to assess whether there were differences in pupil measures between light luminance levels.

There was no significant difference in baseline pupil size between the different luminance conditions, $F(3) = 0.875$, $p=0.416$.

A repeated measures ANOVA showed there to be no significant effect of luminance level on the mean difference in the amplitude of the pupil constriction in response to the light flash, $F(3) = 0.875$, $p=0.416$.

A repeated measures ANOVA found a significant difference in the time taken to reach maximum pupil constriction between light levels, $F(3) = 12.292$, $p<0.001$. Time taken was significantly longer for light level 1 compared to light levels 3 ($p<0.001$) and 4 ($p<0.001$) and light level 2 and level 4 ($p=0.007$) but there was no significant difference between levels 1 and 2 ($p=0.342$), no difference

between level 2 and 3 (p=0.243) and no difference between level 3 and level 4 (p=0.457) see Figure 17.

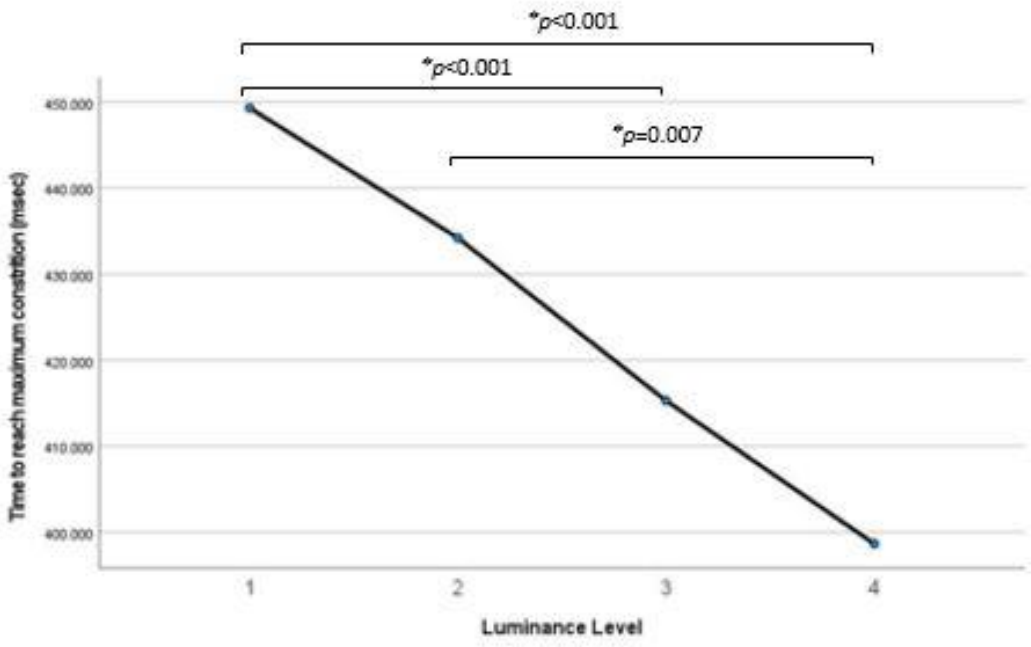


Figure 17. Graph to show mean time taken for pupil to reach maximum constriction in response to different luminance levels.

3.2.9.1 Correlating LC-CR data with pupillometry data

LC-CR was not associated with any of the pupil measures at any of the luminance levels once a Bonferroni correction for multiple comparisons was applied (p=0.00417). However there was a trend for LC-CR to be negatively correlated with baseline pupil diameter and the difference in pupil size in response to light. Correlation values are shown below in Table 8.

Table 8. Pearson correlation coefficient of each pupil measure with LC-CR.

Pupil measure	*r* value	*p* value
Level 1 Baseline pupil diameter	-0.329	0.033
Level 1 Amplitude	-0.319	0.040
Level 1 Constriction velocity	-0.352	0.022
Level 2 Baseline pupil diameter	-0.318	0.040
Level 2 Amplitude	-0.315	0.042
Level 2 Constriction velocity	-0.380	0.013
Level 3 Baseline pupil diameter	-0.327	0.035
Level 3 Amplitude	-0.341	0.027
Level 3 Constriction velocity	-0.250	0.110
Level 4 Baseline pupil diameter	-0.338	0.031
Level 4 Amplitude	-0.372	0.017
Level 4 Constriction velocity	-0.305	0.053

No significant correlations highlighted with *p*-value ≥0.0047 (Bonferroni corrected for multiple comparisons)

3.2.10 Differences in peripheral inflammatory markers between AD and controls

Data for serum APOE4 were normally distributed. An Independent Samples t-test found no significant difference in serum APOE4 levels between the control and AD groups, $t(36) = -0.588$, $p=0.560$ and ANOVA found no difference between control, mild AD and moderate AD groups, $F(2,35) = 0.480$, $p=0.623$. APOE4 concentration was not correlated with LC-CR either overall, $r = -0.286$, $p=0.081$ or in the control group, $r = -0.235$, $p=0.318$ or AD group, $r = -0.311$, $p=0.209$.

Data for serum levels of TNFα were normally distributed and Independent Samples t-test, found no significant difference in TNFα levels between the control and AD groups, $t(36) = -0.987$, $p=0.559$.

Date for serum IL-10 were not normally distributed. A Mann Whitney U test showed no statistically significant difference in serum levels of IL-10 between the control and AD groups, $U = 156$, $p=0.483$.

Date for serum IL-6 levels were not normally distributed. A Mann Whitney U test showed no statistically significant difference in serum levels of IL-6 between the control and AD groups, $U = 126$, $p=0.114$.

Table 9.Serum cytokine levels in control, mild AD and moderate AD groups

	Controls (*n*=20)	Mild AD (*n*=9)	Moderate AD (*n*=9)
APOE4	118.56 ±29.32	129.40 ±34.43	118.70 ±20.71
TNFα	1.77 ±0.42	1.48 ±0.61	1.56 ±0.37
IL-10	0.1 [0.07-0.15]	0.07 [0.06-0.13]	0.09 [0.07-0.23]
IL-6	0.45 [0.34-0.61]	0.34 [0.18-0.70]	0.27 [0.23-0.75]

Data presented as either mean ±standard deviation for normally distributed data or median [Interquartile range] for non-parametric data

3.3 Discussion

3.3.1 LC-CR group differences

The main aim of this study was to determine whether NM-MRI could be used as a biomarker to detect LC signal reductions in patients with AD compared to age and gender matched controls. The trend was for the LC-CR (average of the mean signal intensities of the ROIS for the left and right side of the LC, contrasted with the pons as a reference region) to always be lower in the AD group than in the control group across all 4 axial levels sampled, however this difference only met statistical significance on the axial image corresponding to Level 3. This has been the level used to calculate LC signal intensity in other studies, approximately 7mm below the inferior colliculi and represents the middle of the LC, as the signal is typically greatest here [275,280,285,300]. This finding mimics what has been observed in post-mortem studies which show a reduction in LC cell count in AD brains compared to those without cognitive impairment [113,131,132,194,197], with the greatest degeneration often being evident in the rostral/middle aspects of the LC, whilst there is relative sparing of the caudal part of the LC [108,114,199].

At the time of conducting this study there had only been two previous studies examining differences in LC signal on NM-MRI between healthy controls and AD patient groups, one which only included 6 AD participants found no difference [298] and whilst the other study [275] did find a significant difference in LC signal between AD and controls, the controls were not age matched and it was determined that further studies were required to demonstrate whether NM-MRI scans were useful in identifying participants with AD. The results from this study are in support of the study by Takahashi and colleagues and do suggest that it is feasible to detect signal differences in the LC on NM-MRI. Subsequently other studies have been published which are also in support of these findings [301,302]. Unfortunately in this study there was considerable overlap between LC-CRs in the AD and control groups indicating that the LC-CR has low specificity in differentiating the two groups if used as the sole measure for diagnosis.

There was also no difference in LC signal between the mild AD and moderate AD groups in this study. In post-mortem studies, LC cell loss correlates with dementia severity and is evident at the prodromal or MCI stage [113,132]. The trend was for SMMSE score to be associated with LC signal, although there were no other significant associations between LC signal and the other cognitive tests administered. Other imaging studies have also found a reduction in LC signal in AD groups compared to controls that did not correlate with SMMSE score [301]. The differences observed in cognitive test scores between the three groups were as expected as these scales are typically used to assess dementia progression in clinical care. It is unclear therefore whether the lack of differentiation between mild AD and moderate AD groups by LC-CR value, is due to a limitation in the resolution of the NM-MRI protocol to detect LC cell loss through disease progression or whether there was no difference in signal to be detected. Some studies have only found an association between LC integrity and sub-sets of cognitive tests [315] and it has been highlighted by other researchers that a better understanding is needed of tests that activate the LC [293]. There was a significant reduction in LC signal between the control group and mild AD group suggesting that LC signal loss occurs early in the disease process (again corroborating findings from post-mortem studies). There have been mixed findings as to how early the attenuation in LC signal can be observed, with one study showing a reduction in signal between healthy controls and those with MCI [275] and another study not finding any difference between these two groups [273]. This warrants further research to test the sensitivity of this protocol to detect changes between control participants and those with mild cognitive impairment.

In contrast to previous studies no differences were observed between AD and control participants in measurements of sleep quality and quantity [316]. Additionally, LC signal was not associated with sleep either. The tool used to capture data on sleep looks at current sleep quality and not sleep over the lifetime and as AD pathology begins many years prior to diagnosis, it may be that a better tool assessing sleep at this point may have been more relevant.

3.3.2 Age

There was no effect of age on the LC signal detected in this study despite sampling through the rostral-caudal extent of the LC. The largest NM-MRI study (*n*=605) examining age-related LC signal changes found overall LC-CR signal to increase with age until approximately the age of 60 and then detected a reduction in signal in the rostral part of the LC, though there was greater variation in LC signal in older participants [283]. However there have been mixed findings in post-mortem studies when it comes to age related differences in LC integrity with studies employing more robust methodology often not finding neuronal loss in older adults [109,111,114,123]. Early post-mortem studies showing LC cell loss in aging may have included participants with preclinical AD as

less was known about AD then and people were often diagnosed much later in the disease process [317]. Additionally some other studies have only observed age effects when comparing young and old subjects, rather than looking at age over the lifespan. Participants in this study were all older with the average age being 72 years (± 6years) and the youngest being 57 years at the time of the MRI. A recent imaging study which used participants with a narrow age range also found no effect of age on LC signal in controls [301]. Lastly post-mortem studies have shown considerable variation in LC cell number between individuals [114] and a greater sample size is therefore needed to be able to make any accurate conclusions about LC neuronal loss with age.

3.3.3 Gender

Interestingly a significantly lower LC signal was observed for females compared to males in this study in both the control and AD groups, even after correcting for age. Another study using similar methodology for obtaining measures of LC signal on NM-MRI has also shown this gender effect for women [286], but other studies have not found any gender differences [277], including a study with a larger cohort [283]. Likewise, this does not echo post-mortem counts of LC volume which do not show gender differences [114].

3.3.4 Physiological measures of autonomic function

Three tasks previously shown to evoke reliable changes in blood pressure were chosen as measures of indirect LC activity, Orthostatic reflex, Serial Subtraction and Cold pressor test. It was thought that if the LC is impaired in AD that AD participants would perform differently on these tasks and that they may be associated with LC signal on MRI. Disappointingly none of the tasks elicited the expected response and there were no differences between controls and AD participants on the tasks. There was a decrease in both systolic and diastolic blood pressure on standing from a previous supine position, however it was not to the magnitude that has previously been described and both controls and AD participants had similar minimal decreases. This clearly suggests that physiological measures of autonomic function cannot be used to reliably infer LC signal. The serial subtraction task may have failed because the task was not sufficiently challenging. Ethical approval for the cold pressor test was not gained until after recruitment had started and therefore 16 participants had this procedure omitted from their visit and so sample size was smaller. Nevertheless in those that did participate the expected increase in blood pressure and heart rate did not occur.

3.3.5 Pupillometry

Overall, AD participants had a smaller average baseline pupil size compared to control participants but this was only shown to be significantly different when measured before the light stimulus at luminance level 2. A smaller resting pupil size suggests a deficit in sympathetic innervation in line with LC cell loss [171]. The amplitude of pupil constriction in response to light was reduced in AD participants for all the light flash conditions, however again this difference only reached significance for luminance level 2. This is in line with previous research, but reflects deficits in parasympathetic innervation [318,319]. The time taken for the pupil to reach its maximum constriction i.e., the speed at which it constricted in response to the light flash did not differ between groups. The justification for including pupil measures in this study was to see if indirect measures of LC signal were as effective at distinguishing AD from controls as NM-MRI, potentially replacing the expensive MRI procedure in the future. Unfortunately the pupil response to light was not sufficiently different between AD and controls at all light levels and none of the pupillometry measures were associated with LC signal on the NM-MRI and therefore could not be used in place of NM-MRI to diagnose AD.

Baseline pupil size did not differ significantly in the 25 seconds preceding the light flash at any of the light levels. This indicates that the experiment worked well in that there was enough time for the pupil to dilate again following the light flash for each trial. The time taken for the pupil to reach maximum constriction was significantly longer when the light flash was brighter indicating that the luminance levels did differ from one another. However, the luminance level of the flash did not affect the amplitude of the pupil constriction. This differs to previous studies [320] and suggests that the luminance levels were not sufficiently different to each other to elicit different responses. This may be because the light stimulus came from a computer screen rather than another source and may indicate a limitation in using an eye tracker to capture pupil size, as others have used infra-red television pupillometers [171]. Additionally it has been recommended that caffeine and alcohol should be withheld for at least 3-4 hours prior to testing as this may have an effect on pupil size [312]. Unfortunately this is not something that was controlled for in this study and may have confounded results. Data regarding latency, i.e., the time between onset of the stimulus and the onset of response and recovery time, i.e., the time for the pupil to return to 75% of its size were unfortunately not calculated and may have been better indicators of impairments in sympathetic function.

3.3.6 Limitations

A general limitation of the methods used to calculate a value for LC signal intensity is that rather than being able to generate an absolute value for the LC signal, a contrast ratio is calculated using the pons as a reference region. Although this region was selected because it is the most frequently used region to compare LC signal intensity against [279], some have argued that this region is also subject to age-related decline or undergoes neurodegenerative changes in AD and therefore may not be a reliable region to use [286,287]. This study did not find differences in pons signal between AD and controls proving that the observed differences in LC-CR were due to differences in LC signal, not due to differences in pons signal. Similarly not all studies have seen variation in pons signal between controls and AD participants [280].

This study employed a manual method to calculate LC-CR for each individual participant. Whilst this is typical of the methodology used in previous studies and is more precise than using a probabilistic LC reference atlas, it is time-consuming. Therefore, even if NM-MRI is sensitive enough to be used for diagnosis, the lengthy processing time will restrict its' clinical use. Additionally, images were obtained on a 3T scanner, which are currently not readily available in all hospital settings and the scan has a fairly long acquisition time. Two of the AD participants had to be excluded due to motion artefacts which interfered with calculating a reliable LC signal. Unfortunately remembering to keep still is a problem with this patient group, made more difficult because of the length of the scan.

There is still debate in the field over whether TSE or GRE scans with MT contrast should be used for obtaining optimal images and uncertainty surrounding the source of the neuromelanin contrast observed in NM-MRI studies and therefore data must be interpreted with caution until there is greater clarity over this [274]. This study used a TSE sequence as had most commonly been used previously [279]. However, studies published after data collection for this project had started found GRE sequences with MT contrast to give better spatial resolution than the TSE sequence used in this study both when imaging the LC [272,321], and when imaging the SN [321] though only small sample sizes were used (*n*=6 and *n*=11 respectively). However a previous study which had an equally small sample size did not find the MT sequence to be more sensitive to LC signal than the TSE sequence [282], though the MT sequence did have the advantage of having a lower specific absorption rate. This would be worth considering if using NM-MRI sequences to study the LC in future studies as greater resolution may aid correlating LC signal with dementia severity. Agreeing on a reliable scanning protocol is key if it is to be used reliably in clinical care and to aid comparison between different studies.

Some researchers have imaged the LC at 7 Tesla (7T) which gives greater resolution [271,272], however there is only limited availability of 7T MRI scanners in the UK and so if this type of imaging is to be useful in clinical practice as a biomarker for diagnosis/disease progression, the LC signal changes would need to be detectable at 3T and preferably 1.5T. It was disappointing that the LC signal did not correlate with dementia severity and that a significant difference in signal could not be observed between those with mild AD and those with moderate AD and it is unclear if improved resolution would have enabled differentiation between these groups.

A limitation of this study was its cross-sectional design with no follow up of the participants after they completed the study. Though all control participants scored within the normal range on cognitive assessments when enrolled into the study, it is not known whether they had any preclinical LC neuronal loss which may have affected the study results. No repeats of the MRI scans were obtained, and it would be of interest to conduct a longitudinal study in future to observe any changes in LC signal on NM-MRI within subjects over time. Furthermore no additional biomarkers for AD were obtained, e.g. amyloid or tau load and so the relationship between LC signal on NM-MRI and amyloid and/or tau pathology could not be examined in this study. Many clinical studies use these biomarkers to ensure correct enrolment of AD participants into the stud. All AD participants enrolled in this study had already been diagnosed with AD by a clinician separate to the research study, provided a full clinical history supportive of their diagnosis and were determined to have a typical presentation of AD supported by psychometric testing conducted as part of this study, but amyloid biomarker status was not obtained.

Though the sample size used in this study was powered for the study and similar to that used in other studies [286,293,296,301,322], considerable individual variation in the LC signal suggests that a bigger cohort may be needed to assess how substantial any differences between the groups truly are. However some published studies have used as few as 6 participants to examine LC signal on NM-MRI [281,282].

This study cannot make any comments regarding lower cortical NA concentration in AD patients as no measure of NA concentration was obtained. NA concentration would need to be determined indirectly from CSF samples which would have been far more invasive for the participant and beyond the scope of this project. Nevertheless, it is an area of great interest for future work.

In conclusion, LC signal was found to be reduced in people with AD compared to age and gender matched controls and NM-MRI is a promising, non-invasive and reproducible technique which will be able to differentiate these groups. It is likely the reduction in LC signal on NM-MRI reflects LC degeneration as observed in post-mortem studies of these populations. However as this is a

clinical study there is no way to be sure of the biological processes underlying this observation and even if there was funding and consent to follow up these participants until death for histopathological analysis this could be years, possibly decades and would not be reflective of the images obtained now as part of this study. LC signal is not able to tell us anything about the functional ability of the LC or any resulting synaptic loss. Whilst studies are beginning to use functional MRI to assess the connectivity of the LC to other brain regions and look at how this is affected in AD, the small size of the LC makes this challenging. This study supports other NM-MRI studies and has the advantage of having robust inclusion criteria, only including those with a clear clinical diagnosis supported by psychometric testing and excluding those taking SNRI's. Furthermore, none of the participants were smokers and so any effects of LC activation by nicotine did not need to be considered when performing the data analysis [292]. It is the first study to correlate structural NM-MRI scans with pupillometry and other indirect physiological measures of LC activity and is the largest individually matched case-control study in this patient group. As test-retest reliability of NM-MRI scans is known to be good [289,291], future work should aim to look at longitudinal changes in LC signal and to determine how early signal attenuation can be detected in those with cognitive impairment in order to further assess its effectiveness as a biomarker for AD and clinical use in diagnosis.

Chapter 4 Investigating the relationship between LC cell loss and inflammatory changes and extra-cellular NM deposits

4.1 Method

4.1.1 Human brain samples and ethics

The South West Dementia Brain Bank (SWDBB) in Bristol provided the human brain tissue used in this study funded by Alzheimer's Research UK (ARUK-PPG2018B-019). 60 cases were selected based on their Braak staging of tau pathology, a marker of AD severity. The cases were divided into three groups: 20 cases Braak stage 0-II, 20 cases Braak stage III-IV and 20 cases Braak stage V-VI. For each case, frozen tissue and 20 formalin-fixed, paraffin embedded (FFPE) sections of 6µm thickness were provided from two brain areas: the pons at the level of the rostral/middle LC and the middle superior temporal gyrus. It was decided to examine the rostral middle portion of the LC to explore changes within the LC throughout disease progression as previous studies have shown relative sparing of caudal LC neurons to neurodegeneration in AD and that the rostral/middle areas project to the hippocampus and temporal cortex. The middle superior temporal gyrus was selected because it is an area where the LC projects to, but does not receive input from, making it a suitable area to examine the relationship between changes seen in the LC and cortical changes, throughout disease progression. For 8 of the cases, frozen tissue was not available so only the FFPE slides were provided. For one of the control cases (Braak stage I, case 803) only frozen tissue was available and no FFPE slides were provided. Braak staging, post-mortem delay and other anonymised clinical and histological information were provided by SWDBB and are provided in Appendix E.

Cases were preferably selected that had a post-mortem delay of less than 72 hours and from formalin blocks processed at the time of the original post-mortem examination as fixative can impair immunodetection. Cases were excluded if there was significant co-existing vascular disease or any disease or insult leading to immune activation as this would confound the results.

The study was performed under the ethical approval from SWDBB, provided by the South West Central Bristol NRES committee (REC reference: 08/H0106/28+5).

The study was registered with the University Ethics and Governance Online (ERGO, ref 47335) prior to the study start date.

All methods and reagents used were risk assessed in line with the University of Southampton's Health and Safety regulations.

4.1.2 Power Calculations

Previous studies of microglia in post-mortem tissue by the Boche group have found that a sample size of at least 30 is sufficient to demonstrate findings. A sample size of 20 in each group with a standard 80% power and 5% significance level will be able to detect standardised effect sizes of at least 0.443. Previous studies reveal a range of standardised effect sizes from 0.65 (difference of 20.6 with a standard deviation of 31.6) to 1.8 (difference of 0.18 with a standard deviation of 0.1) so this study should be adequately powered to pick up reasonable effect sizes.

4.1.3 Immunohistochemistry

Immunohistochemistry (IHC) experiments were performed in the Histochemistry Research Unit, University of Southampton. 118 slides per antibody were tested (59 cases of both temporal and pons tissue), typically split into 3 runs due to the volume of slides. Cases from each Braak stage were split equally between the runs to minimise artefacts that could hinder comparison. However, for each case both brain sections, temporal and pons, were included within the same run to ensure the results from each area were comparable to each other.

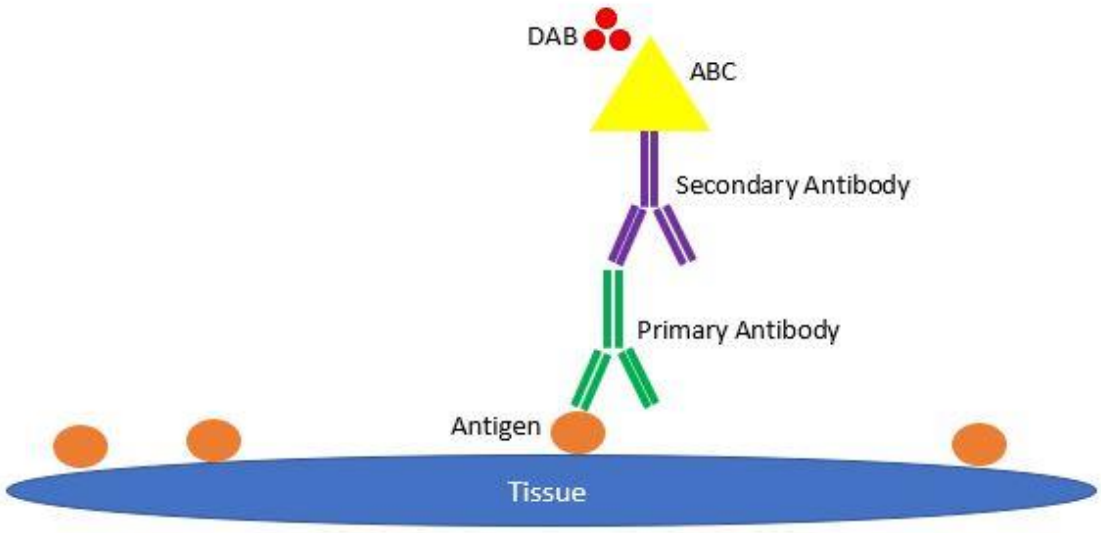


Figure 18. Immunohistochemistry experiment schematic

FFPE slides were immersed in a series of tissue tek tissue clear solutions (Sakura) and then rehydrated through graded alcohols and brought to distilled water. They were then treated with 10% concentration hydrogen peroxide (Sigma) for 21 hours to both block endogenous peroxidase activity and to bleach the neuromelanin as it is a similar brown colour as the chromogen used to visualise the protein of interest and made it difficult to assess protein load. Optimisation of the bleaching protocol is shown in Appendix F. The sections were washed using a Tris-buffered saline (TBS) solution between each step. The appropriate antigen retrieval method for each primary antibody was performed as listed in Table 10, followed by use of a blocking solution, typically 5% normal goat serum (2B Scientific), to saturate the samples. Primary antibodies were incubated for either 90 minutes at room temperature or overnight at room temperature. Biotinylated goat anti-rabbit or anti-mouse secondary antibodies (2B Scientific) were used at a concentration of 1:800 for 30 minutes at room temperature and bound antibodies were visualised using the avidin-biotin-peroxidase complex (ABC) method (2B Scientific) with 3,3' diaminobenzidine (DAB) (2B Scientific) as the chromogen. All sections were then counterstained with haematoxylin for 30 seconds, dehydrated and mounted using Pertex (CellPath). The full protocol is listed as Appendix D.

4.1.4 Antibodies

Experiments to optimise the concentrations of most of the antibodies and antigen retrieval methods were completed by the Boche laboratory prior to this study with the exception of DβH and TH which I optimised before use. The following primary antibodies were selected:

- 4G8 (BioLegend), monoclonal antibody diluted at 1:2000 used to visualise Aβ deposition.
- AT8 (Thermoscientific), monoclonal antibody made in mouse which recognises phosphorylated paired helical filament human tau which is phosphorylated at serine 202 and threonine 205. This antibody has been shown to be a reliable method of examining hyperphosphorylated tau in human brain tissue regardless of fixation time length [14] enabling early detection of tau pathology.
- Iba1 (Wako), ionized calcium binding adaptor molecule 1, polyclonal antibody to detect a marker of microglial homeostasis associated with cell motility.
- HLADR (Dako), a marker of activated microglia related to the antigen-presenting function of microglia
- CD68 (Dako), a marker of activated microglia expressed by phagocytic microglia
- DβH (Abcam), monoclonal antibody made in rabbit which recognises dopamine-β-hydroxylase and used to visualise noradrenergic neurons and processes

- TH (Abcam), recombinant monoclonal antibody made in rabbit which recognises tyrosine hydroxylase and therefore used to visualise dopaminergic and noradrenergic neurons and processes.

Table 10. Primary antibody details including host, company, antigen retrieval method, dilution and incubation time and length of time needed for chromogenic reaction.

Antibody	Host	Company	Dilution	Antigen Retrieval	Incubation	DAB Time (min)
4G8, Monoclonal	Mouse	*Covance-BioLegend*	1:2000	100% formic acid and microwave citrate buffer	90' RT	2.5
AT8, Monoclonal	Mouse	*Thermoscientific*	1:500	Microwave citrate buffer pH6'	O/N RT	5
Iba1, polyclonal	Rabbit	*Wako*	1:1500	Microwave citrate buffer pH6	O/N RT	3
HLA-DR	Mouse	*Dako*	1:200	Microwave citrate buffer pH6	O/N RT	6.5
CD68	Mouse	*Dako*	1:500	Microwave citrate buffer pH6	O/N RT	5.5
DβH, Monoclonal	Rabbit	*Abcam*	1:1500	Microwave citrate buffer pH6	90' RT	5
TH, Monoclonal	Rabbit	*Abcam*	1:1500	Microwave citrate buffer pH6	90' RT	2

4.1.5 Fontana-Masson stain

Neuromelanin was visualised using the Fontana-Masson stain kit from Abcam (ab150669) by following the staining protocol in the datasheet. First Ammoniacal Silver solution was prepared by mixing 54ml of distilled water with two vials of the Silver Nitrate Solution (10%, 18ml) in clean glassware. Concentrated Ammonium Hydroxide was added one drop at a time turning the liquid a dark brown; the liquid was swirled gently after each drop until the mixture was transparent and all the sediment dissolved. The liquid was then warmed in a 60°C oven. Locus coeruleus slides were deparaffinised in a series of tissue tek solutions and then rehydrated through graded alcohols and brought to distilled water in batches of 16 slides. They were incubated in the Ammoniacal Silver Solution for 40 minutes. Slides were rinsed in distilled water before being incubated in Gold Chloride Solution (0.2%) at room temperature for 30 seconds. Slides were rinsed again in distilled water and incubated in Sodium Thiosulphate Solution (5%) for 1.5 minutes at room temperature. Slides were then rinsed in running tap water for 2 minutes followed by 2 changes of distilled water. Lastly slides were incubated in Nuclear Fast Red Solution for 5 minutes

and again slides were rinsed in running tap water for 2 minutes followed by 2 changes of distilled water. Slides were quickly dehydrated in fresh Absolute Alcohol and mounted.

4.1.6 Quantification

All images were scanned using the Olympus VS-110 slide scanner at x20 magnification. One FFPE slide from each case and brain area was stained with haematoxylin and eosin (H&E) to enable identification of cell structures. For the temporal cortex sections an appropriate sulcus was identified and marked on the H&E slides by a neuropathologist. This area was used as a reference point so that the same area of the sulcus was sampled for each antibody. 30 square ROIs of 500µm² at magnification x20 were placed in a zig-zag pattern along the grey matter of the sulcus. The zig-zag pattern was used to ensure unbiased sampling from all layers of the grey matter. An example is shown below, see Figure 19. For the pons sections, a singular ROI of 1.67mm² was placed over the centre of the LC which was easily identifiable for most cases on the H&E slides by the large pigmented cells. An ROI of 1.67mm² was chosen for this area because it is the same size as the ROI used in the clinical NM-MRI study (Chapter 3) and provided adequate coverage of the LC usually in its entirety, example shown below, Figure 20.

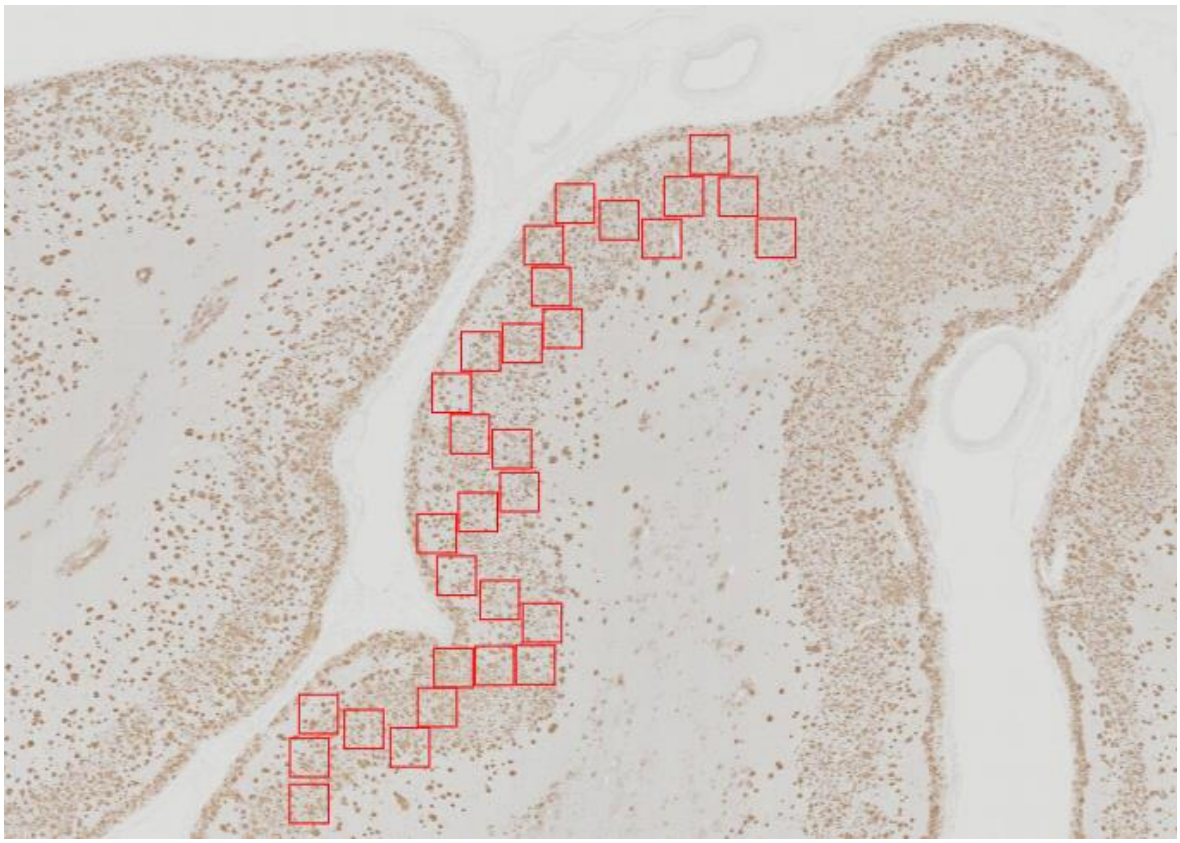


Figure 19. Example of placement of ROIs on a temporal cortex section.

For this case ROIs were placed in a similar zigzag orientation in the same region of sulcus for each antibody tested.

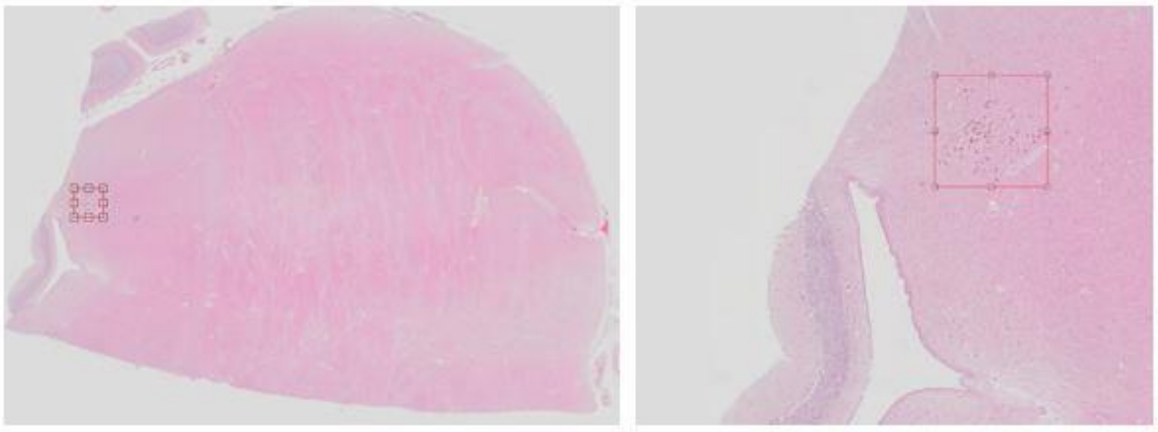


Figure 20. Example of placement of ROI over the LC on a pons section

The ROIs were analysed using Fiji ImageJ, v1.52p software, a Java-based imaged processing program [323]. For each ROI, the background was subtracted and the red and green colours were removed from each image using a 'RGB recolour' plugin. Images were then converted to 8-bit black and white images. Cases that showed extremes in staining i.e., those that had the lightest or those that had the most intense staining were used to set a threshold that incorporated all protein staining but did not include aspecific staining. The same threshold was applied to all ROIs for the same antibody for each brain area. A mask was then created for each ROI to show the staining only in a black and white image, based on the threshold. The percentage area covered by the staining was calculated using the area fraction measure function, to provide a protein load percentage for each ROI. These steps are shown below in Figure 21. A macro was written (Figure 22) to automate the calculation of percentage of staining, expressed as protein load, from each ROI. For each antibody, a sample of the black and white images were compared with their original digital images to check the macro had performed the task correctly. Quantification of images was performed blind to Braak stage group and clinical diagnosis.

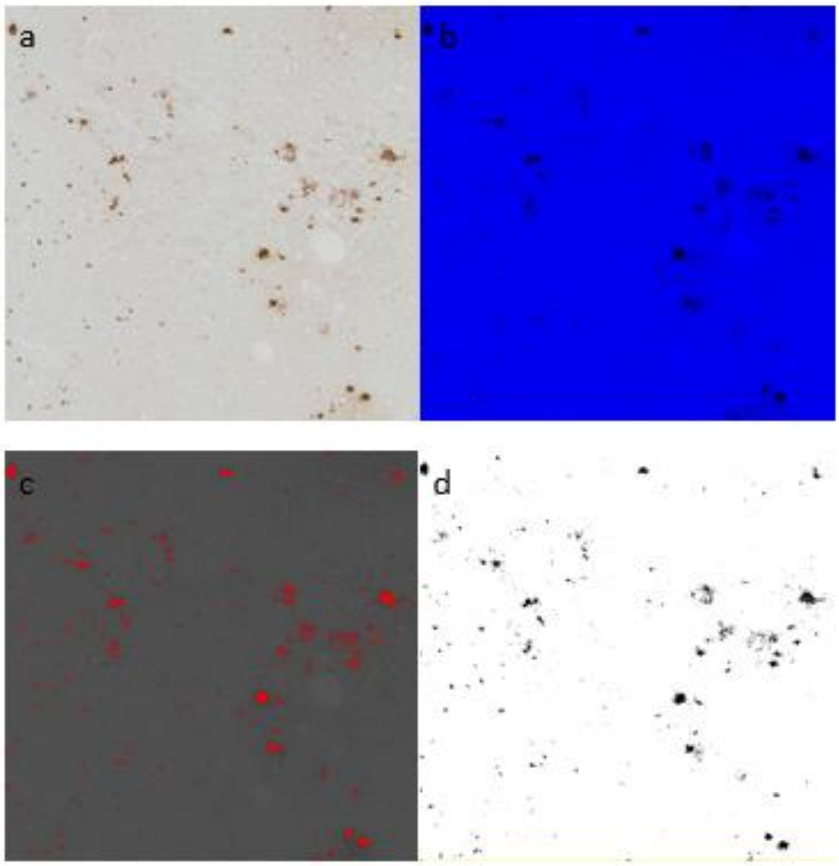


Figure 21. Processing steps to calculate percentage staining load with ImageJ

a) background was subtracted, b) Blue colour extracted using RGB recolour plugin, c) converted to 8-bit image, threshold applied to detect staining only, d) black and white mask created. Measure area fraction used to determine the percentage covered by black in the mask reflecting the percentage protein load

```
function processImage(path) {
    if (endsWith(path, ".tif")) {
        open(path);
        Title=getTitle();
        run("Subtract Background...", "rolling=50 light");
        run("RGB Recolor", "red_factor=0 red_constant=0 green_factor=0 green_constant=0 blue__factor=1 blue_constant=0");
        run("8-bit");
        setAutoThreshold("Default dark");
        setThreshold(0, 54);
        setOption("BlackBackground", false);
        run("Convert to Mask");
        run("Set Measurements...", "area_fraction display redirect=None decimal=3");
        run("Measure");
        saveAs("TIFF", path+"_BW");
        close();
```

Figure 22. Macro used to automate processing steps shown in Figure 21.

For 3 of the LC cases (#751, 826 and 856) the pons had been sliced in a way that the LC was close to the edge of the tissue and was partially missing. Rather than excluding these cases, the ROI was still placed over the centre of the LC, meaning that some of the ROI was taken up by empty space. Therefore, for these cases, the area of the tissue was calculated, and the protein load

percentage adjusted to reflect the percentage area of tissue stained rather than the percentage area of the entire ROI stained. Examples are shown below in Figure 23.

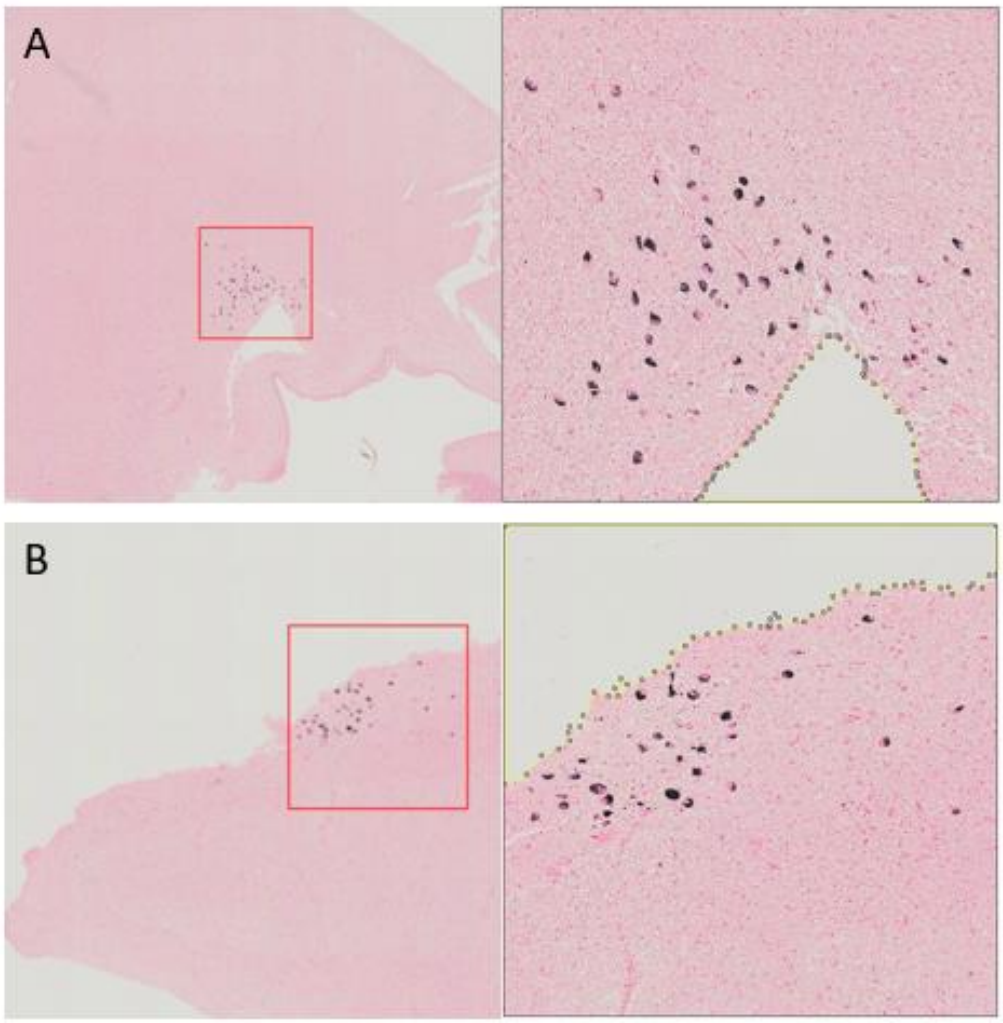


Figure 23. Illustration of placement of ROI over Locus coeruleus where tissue is missing.

In Case 751 (A) and Case 826 (B), some of the tissue containing LC cells is missing. Therefore, the area of empty space is subtracted from the total tissue area and the percentage of staining is adjusted so it is a percentage of the tissue in the ROI and not the total ROI area.

4.1.6.1 Quantification of neuropil degeneration

Neuropil degeneration was quantified in a similar way to protein expression above using the H&E slides for both brain areas. With this stain, the threshold measured the percentage area that was unstained, and the value was compared across Braak groups.

4.1.6.2 Quantification of cell number

The LC sections stained using the Fontana-Masson kit were also stained with Nuclear Fast Red which enabled visualisation of some cell structures. On the Fontana-Masson stained sections in which LC cells were easily identifiable due to neuromelanin pigment, regions of interest were drawn around each LC cell (Figure 24). Only cells in which a significant portion of the cell body was evident, with a minimum area of 2000 pixels, were included and counted and then the total was compared across the three Braak groups. It was not a requirement for the nucleolus to be visible as often this was obscured by the neuromelanin pigment.

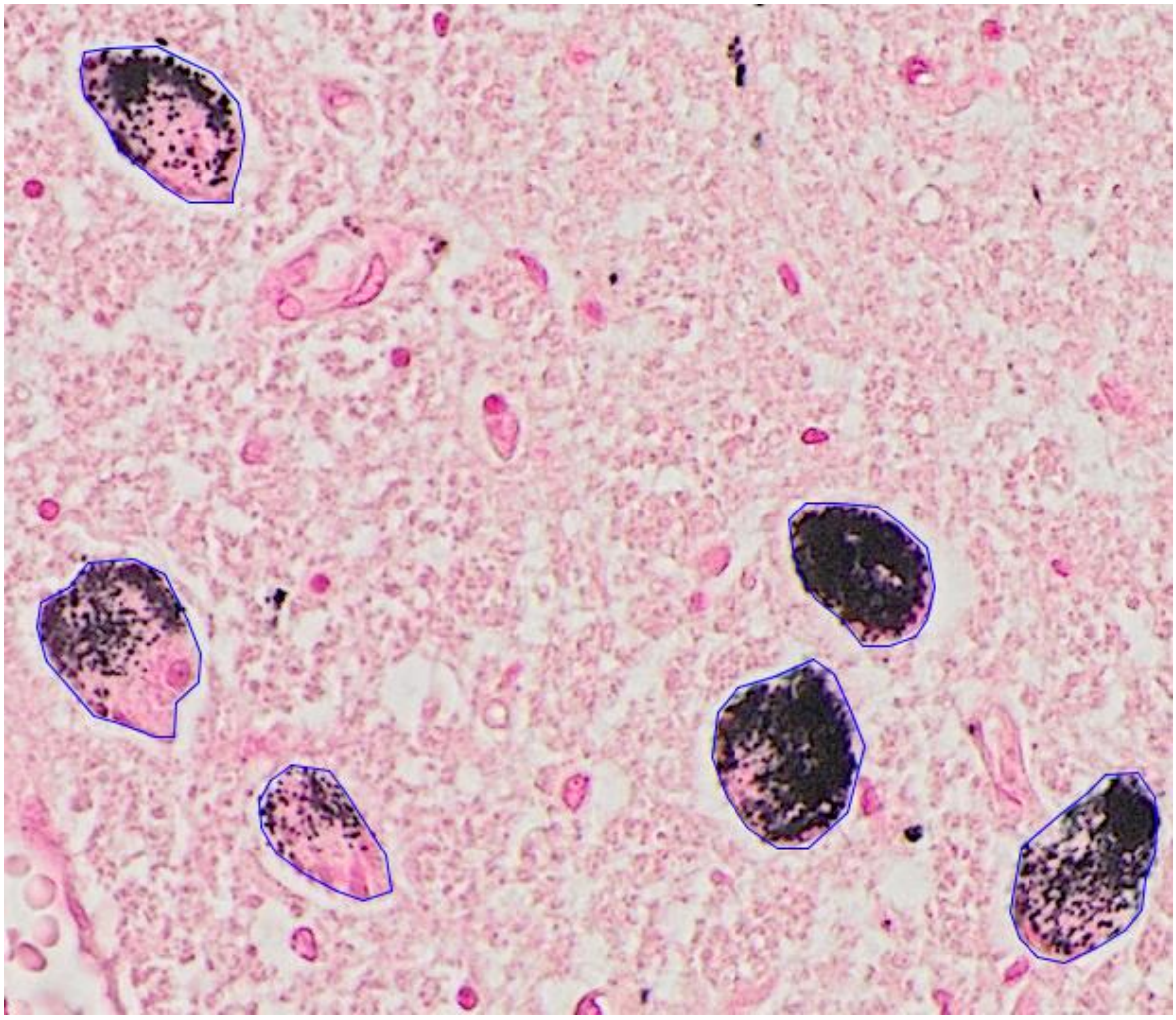


Figure 24. Example of ROIs drawn around LC cells (blue) containing neuromelanin pigment (black) on Fontana-Masson stained section

(Image from case 751, Braak stage 0)

Additionally, differences between the average size of the LC cells and the shape of LC cells were also compared across Braak groups.

4.1.6.3 Quantification of extraneuronal neuromelanin

Whilst it was clear that some NM staining was within the LC cell bodies, often smaller NM particles were also observed and it was difficult to determine whether these were in the extracellular space, had been phagocytosed by microglia or were contained within parts of LC cells in which the cell body was not visible. As NM typically accumulates in the cell body and not in the axons or dendrites of LC cells, the smaller NM particles are unlikely to be from neuronal processes. Two methods of calculating extraneuronal NM were undertaken. The first was an automated method in which the percentage area of all NM not contained within the LC cell ROIs (already drawn to quantify cell number) was calculated and compared across the 3 Braak groups. Secondly a manual method was performed in which ROIs were drawn around all larger particles of NM that appeared to be within LC cells whether fully or partially visible. NM within all these ROIs was excluded from the total amount of NM stained to give a revised total of extraneuronal NM, compared across the 3 groups. This method was thought to give a more accurate, though less reproducible, total extraneuronal NM calculation.

4.1.7 Frozen tissue for Meso Scale Discovery

Frozen tissue from both the temporal cortex and the pons to include the LC was provided for 52 cases out of the 60 cases used for IHC. Meso Scale Discovery (MSD) biomarker immunoassay kits were used to measure the levels of various inflammatory analytes within the tissue.

4.1.7.1 Homogenisation of brain tissue

For the temporal region, approximately 100mg of tissue was cut from the frozen sample provided by the SWDBB and inserted into a 2ml test tube. The remaining frozen tissue was re-frozen for potential use in future projects. For the pons sections, it was not possible to cut these sections down to 100mg and be sure they still contained the whole of the LC and therefore all the frozen tissue provided by SWDBB was used. Lysis buffer, made by combining 10ml of RIPA buffer with a combination tablet of protease and phosphatase inhibitors, was added to each sample at a dilution of 500µl of buffer to 100mg of tissue. Brain tissue from each case was weighed so that the exact amount of lysis buffer required could be calculated before adding it to the tissue samples. Homogenisation beads were added to the mixture and the sample placed in a bead microtube homogeniser for a minimum of 40 seconds until samples appeared to be completed homogenised and no solid tissue remained. The tubes were then centrifuged at 4°C for 30 minutes at 13,000 rpm. The tubes were kept on wet ice and the supernatant for each sample was promptly pipetted off and divided into 3 aliquots with 100µg for the MSD assay, 20µg for the BCA assay and the rest into a spare aliquot to be used in future projects. All samples were then frozen

at -80°C. The pellet which also contained the beads was discarded. The full protocol for the homogenisation of the frozen tissue is detailed in Appendix G.

4.1.7.2 Mesoscale Discovery Assay

Three MSD V-PLEX immunoassay pre-configured kits were selected:

- V-PLEX Proinflammatory Panel 1 Human Kit – to detect IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α.
- V-PLEX Cytokine Panel 1 Human Kit – to detect GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, and VEGF-A.

These 20 cytokines are involved in the inflammatory response and immune system regulation and have been implicated in AD (as well as other disorders). A summary of the role of each of these markers is outlined in Table 11 and Table 12.

- V-PLEX Chemokine Panel 1 Human Kit – to detect eotaxin, eotaxin-3, MIP-1β, MIP-1α, TARC, MCP-1, MDC, MCp-4, IP-10 and IL-8.

These 10 chemokines which aid the migration of immune cells via chemotaxis are associated with AD (as well as other disorders). A summary of the role of each of these markers is outlined in Table 13.

These kits each contained pre-coated plates to enable a fast, convenient, validated way to measure the levels of multiple targets within the same sample.

The homogenised brain samples were provided to Dr Laurie Lau who performed the MSD assay using a Meso Quickplex SQ120 plate reader and provided the results for each marker for each case in pg/ml.

The protein level values were normalised to the total protein concentration obtained by the BCA Assay.

Table 11. Summary of the function of each of the markers examined as part of the V-PLEX Proinflammatory panel

Marker	Function
IFN-γ (Interferon gamma)	• activated by Interleukin-12 and secreted by type-1 helper and cytotoxic T cells • activates macrophages and induces MHC Class II expression • Imbalance in IFN-γ associated with autoimmune diseases
IL-1β (Interleukin 1 beta)	• secreted by activated macrophages/microglia • induces IL-2 release, B cell maturation and proliferation • Induces production of prostaglandins (along with IFN-γ, IL-6, TNF-α)
IL-2 (Interleukin-2)	• also known as T cell growth factor, produced by T cells and involved in T cell proliferation and differentiation • helps T cells distinguish between self and foreign • can readily penetrate the blood-brain barrier so could enable communication between CNS and peripheral
IL-4 (Interleukin-4)	• also known as B cell stimulatory factor 1, produced by mast cells, helper T cells, eosinophils, and basophils • induces differentiation of naive T cells into helper T cells, helper T cells then produce more IL-4 in a positive feedback loop. • similar functions to IL-13
IL-6 (Interleukin-6)	• also known as B cell stimulatory factor 2 • secreted by macrophages in response to pathogen associated molecular patterns (PAMPS) • induces neutrophil production in bone marrow • Associated with fever and the acute phase response
IL-8 (Interleukin-8)	• induces chemotaxis primarily in neutrophils, but also basophils and T cells
IL-10 (Interleukin-10)	• also known as cytokine synthesis inhibitory factor • typically anti-inflammatory cytokine, downregulates helper T cell cytokines and inhibits IFN-γ, IL-2, IL-3, TNF-α, TNF-β and GM-CSF • aids B cell survival, proliferation and antibody production
IL-12p70 (Interleukin-12p70)	• secreted by macrophages and T cells • activates cytotoxic T cells and NK cells to stimulate production of IFN-γ
IL-13 (Interleukin-13)	• secreted by Type 2 helper cells, NK cells, mast cells, basophils and eosinophils • similar function to IL-4 • regulates B cell proliferation • associated with allergic inflammation, asthma and allergic rhinitis
TNF-α (Tumor Necrosis Factor alpha)	• mainly secreted by macrophages • stimulates IL-1 • initiates acute phase response, induces fever, and can induce sepsis

Table 12. Summary of the function of each of the markers examined as part of the V-PLEX Cytokine Panel 1

Marker	**Function**
GM-CSF (Granulocyte-macrophage colony-stimulating factor)	• secreted by macrophages • aids in stimulating stem cells to produce granulocytes and monocytes • aids neutrophil migration • involved in dendritic cell differentiation
IL-1α (Interleukin-1 alpha)	• secreted by macrophages and neutrophils • increases blood neutrophils and lymphocyte production in response to infection • induces IL-2 secretion along with IL-1β • induces fever and sepsis
IL-5 (Interleukin-5)	• also known as B cell growth factor and T cell replacing factor • produced by Type 2 helper cells and eosinophils • associated with allergic inflammation, asthma and allergic rhinitis
IL-7 (Interleukin-7)	• hematopoietic growth factor • aids in B cell proliferation and important for B cell and T cell development
IL-12/IL-23p40 (Interleukin-12/ Interleukin-23p40)	• secreted by macrophages, dendritic cells and neutrophils in response to infection • stimulates production of T cells and NK cells as well as IFN-γ • induces differentiation of naive T cells into helper T cells • associated with autoimmune disease
IL-15 (Interleukin-15)	• secreted by macrophages and dendritic cells • stimulates production of T cells and NK cells
IL-16 (Interleukin-16)	• also known as lymphocyte chemoattractant factor, attracts activated T cells • stimulates production of IL-2 and IL-15
IL-17A (Interleukin-17A)	• secreted by activated T cytotoxic and helper cells • key part of the TH17 pathway • stimulates IL-23 production and can stimulate expression of IL-8 and nitric oxide • high levels associated with rheumatoid arthritis and multiple sclerosis
TNF-β (Tumor Necrosis Factor beta)	• also known as lymphotoxin alpha • produced by lymphocytes • cytotoxic for many tumour cells, regulating survival, proliferation, differentiation and apoptosis. • unregulated production can result in creation of tumours
VEGF-A (Vascular endothelial growth factor)	• induces blood vessel growth - angiogenesis, vasculogenesis and endothelial cell growth • promotes cell migration, inducing permeability of blood vessels and inhibits apoptosis

Table 13. Summary of the function of each of the markers examined as part of the V-PLEX Chemokine Panel

Marker	Function
Eotaxin (CCL11)	• ligand for CCR3 • Recruits eosinophils in response to allergens • chemotactic for eosinophils and basophils • Implicated in allergic response, also chronic bronchitis and osteoarthritis
Eotaxin-3 (CCL26)	• ligand for CCR3 • Produced by endothelial cells stimulated with IL-4 • chemotactic for eosinophils and basophils • Implicated in rheumatoid arthritis and allergic asthma
MIP-1β (Macrophage inflammatory protein beta) (CCL4)	• ligand for CCR1 and CCR5. • Produced by macrophages and monocytes after stimulation with LPS or IL-1β • Induces release of proinflammatory cytokines e.g., IL-1, IL-6 and TNF-α • Role in organ transplant rejection
MIP-1α (Macrophage inflammatory protein alpha) (CCL3)	• ligand for CCR1, CCR4 and CCR5 • Induces chemotaxis and activation of granulocytes • Induces fever
TARC (Thymus and activation regulated chemokine) (CCL17)	• ligand for CCR4 • Produced in thymus by antigen presenting cells, e.g. dendritic cells, macrophages and monocytes • Induces chemotaxis in T-helper cells (where CCR4 mostly expressed) • Produced after stimulation with IL-4, upregulated by GM-CSF • Neutralising TARC can relieve inflammatory arthritis
MCP-1 (Monocyte chemoattractant protein 1) (CCL2)	• ligand for CCR2 • Induces chemotaxis in monocytes, basophils, memory T cells and dendritic cells to sites of inflammation • Associated with rheumatoid arthritis and psoriasis
MDC (Macrophage derived chemokine) (CCL22)	• ligand for CCR4 • Induces chemotaxis in dendritic cells, NK cells and activated T-lymphocytes to inflammatory sites. • Implicated in atopic dermatitis. Role in chronic inflammation
MCP-4 (Monocyte chemoattractant protein 4) (CCL13)	• ligand for CCR3 • Induces chemotaxis in monocytes, eosinophils, T cell and basophils • Produced after stimulation with IL-1 and TNF-α • Role in allergic asthma, stimulates histamine release from basophils
IP-10 (Interferon gamma-induced protein 10) (CXC10)	• ligand for CXCR3 • Induces chemotaxis in monocytes and T lymphocytes • Produced after stimulation with IFN-γ • Promotes T cell adhesion to endothelial cells • Implicated in COPD, MS, asthma
IL-8 (Interleukin-8) (CXCL8)	• ligand for CXCR1 and CXCR2 • Known as neutrophil chemotactic factor, induces chemotaxis primarily in neutrophils so they migrate towards infections site, also stimulates phagocytosis once neutrophils have arrived, promotes angiogenesis • can be secreted by any cell with toll-like receptors, mainly macrophages and endothelial cells • Associated with acute pancreatitis

4.1.7.3 Bicinchoninic Acid (BCA) Protein assay

In order to determine the total protein concentration of each sample a BCA assay (Pierce BCA Protein Assay Kit, Thermoscientific) was performed. The 1ml albumin standard (2mg/ml) was diluted into 9 aliquots using the lysis buffer to prepare 9 diluted albumin standards with known concentrations: A (2000µg/ml), B (1500µg/ml), C (1000µg/ml), D (750µg/ml), E (500µg/ml), F (250µg/ml), G (125µg/ml), H (25µg/ml) and I (0µg/ml). On a 96 flat-bottom well transparent Greiner plate, 10µl of each standard was pipetted into a well in triplicate. For the samples, 4µl of each of the homogenised brain samples was combined with 36µl of lysis buffer. 10µl of this sample was pipetted into a well in triplicate. 200µl of working reagent (made from 50 parts of solution A to 1 part Solution B) was then added to each of the wells. The plate was covered in foil and incubated at 37°C for 30 minutes. The plate analysis was conducted using an Infinite F200 PRO plate reader with iControl 1.9 software to produce an Excel document with absorbance values for each well. A total of 5 plates were needed to analyse all the samples. An example of the layout of the plate is shown in Figure 25. The full protocol for preparing the BCA assay is detailed in Appendix H.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2000	1500	1000	750	500	250	125	25	0	Sample 16	Sample 16	Sample 16
B	2000	1500	1000	750	500	250	125	25	0	Sample 17	Sample 17	Sample 17
C	2000	1500	1000	750	500	250	125	25	0	Sample 18	Sample 18	Sample 18
D	Sample 1	Sample 1	Sample 1	Sample 6	Sample 6	Sample 6	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
E	Sample 2	Sample 2	Sample 2	Sample 7	Sample 7	Sample 7	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
F	Sample 3	Sample 3	Sample 3	Sample 8	Sample 8	Sample 8	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
G	Sample 4	Sample 4	Sample 4	Sample 9	Sample 9	Sample 9	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
H	Sample 5	Sample 5	Sample 5	Sample 10	Sample 10	Sample 10	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23

Figure 25. Example layout of the BCA plate.

Each plate has 9 standards repeated 3 times and 23 samples also repeated 3 times.

4.1.7.4 BCA Analysis

For each plate the average of the 3 absorbance values for each standard or sample was calculated. The coefficient of variance for each absorbance value was detected using the

calculation (standard deviation/mean) x 100. Any value greater than 10 was excluded as an outlier and in those instances the average of the remaining 2 absorbance values was used. The known concentrations of the 9 standards were used to make a standard curve graph. The equation for the curve was used to determine the unknown concentrations of the samples. These were then multiplied by 10 as the samples were diluted tenfold when performing the BCA assay.

4.1.8 Statistical Analysis

The percentage protein load of each antibody for the 30 ROIs extracted from the temporal cortex section for each case were averaged to get the mean percentage of staining for that antibody. The percentage protein load of the ROI covering the LC section was also calculated. Statistical analysis of these protein loads was performed using the software IBM SPSS v27 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0.1.0. Armonk, NY: IBM Corp).

Normality of the data was assessed for each variable using histograms, descriptive statistics and Q-Q plots. Where data were normally distributed, analysis of Variance (ANOVA) was used to compare differences in protein load between the different Braak stage groups in both the pons and temporal sections. Where data were not normally distributed the Kruskal-Wallis test was used to compare the markers across groups. Correlation coefficients were used to examine whether age was related to any of the markers and thus to determine whether age corrections were required when looking at correlations between markers studied. As there was a significant association between age and p-tau in the temporal cortex and age was not normally distributed, data for p-tau in the temporal cortex were log transformed and logistic regression analysis was used to correct for age. Pearson's or Spearman's rank correlations were used as appropriate to explore the relationship between markers in the 2 brain areas as well as any relationship between the different markers. A threshold of 5% was used for intergroup comparisons. Bonferroni corrections were applied to adjust *p* values for the correlations and to the Meso Scale Discovery data to correct for multiple comparisons. Adjusted correlations corrected for age and gender did not alter the relationships.

4.2 Results- Immunohistochemistry

The 60 cases were divided into 3 Braak stage groups, (Group 1= Braak stage 0-II, Group 2 = Braak stage III-IV, Group 3 = Braak stage V-VI). For each marker examined as part of this study, differences between the 3 groups are first reported in the LC, then in the temporal cortex, followed by any relationship between the two brain areas at each Braak group. Average values for each Braak group for each marker examined are provided in summary Table 16. When data

were normally distributed and parametric tests performed, results for each group are presented as number of cases(n), mean(m) and standard deviation (SD). Where data were not normally distributed and non-parametric tests were used, results for each group are presented as median and Interquartile range (IQR).

4.2.1 Demographics of the samples

For one case from the Braak stage 0-II group (#803), only frozen tissue was provided by the brain bank and therefore IHC could not be performed for this case. When examining the staining for the LC, case #714, Braak group III-IV, was not included as the pons sections did not contain the LC. For the temporal cortex, case #921, Braak group 0-II was not included in the analysis as the tissue was ischemic and damaged. Table 14 shows a summary of data for age at death, gender and post-mortem delay.

Table 14. Demographic and post-mortem characteristics of the three groups

	Braak group 0-II (*n*=19)	**Braak group III-IV (*n*=20)**	**Braak group V-VI (*n*=20)**
Age at death (years)	85.4 ±8.7	86.2 ±6.4	80.5 ±7.6
Gender	6M, 13F	11M, 9F	9M, 11F
Post-mortem delay (hours)	42.1 [37.8-64.8]	35.5[31.3-49.3]	40.9 [14.5-50.7]

Results are presented as mean ± standard deviation for normally distributed data or median [interquartile range] for non-parametric data. *M* male, *F* Female

Data for age were normally distributed. ANOVA found a significant difference in age between groups, $F(2,56) = 3.339$, $p=0.043$. A Tukey post hoc test found age in Braak group V-VI to be lower than Braak stage III-IV, $p=0.05$, but there was no difference in age between Braak group V-VI and 0-II or between III-IV and 0-II. As there was a significant difference in age between groups, correlation coefficients were used to assess the relationship between age and each of the markers. Correlations between age and AT8 in the temporal cortex, was significant as summarised in Table 15 and therefore AT8 in the temporal cortex was corrected for age, which did not substantially alter the relationship.

Data for post-mortem delay were not normally distributed. There was no significant difference in post-mortem delay between groups, $H(2) = 3.118$, $p=0.210$. There was missing data for one case in Braak group 0-II for this variable.

There was no significant difference in the proportion of males to females across the 3 groups as determined by Pearson Chi-Squared test, $\chi^2(2) = 2.179$, $p=0.336$.

Table 15. Spearman's rank correlation with each marker with age

Marker	Spearman's rho	*p* value
Aβ (4G8) in LC	-0.207	0.13
Aβ (4G8) in TC	-0.164	0.22
p-tau (AT8) in LC	-0.090	0.51
p-tau (AT8) in TC	-0.277	0.04*
Iba1 in LC	0.087	0.54
Iba1 in TC	0.092	0.49
HLA-DR in LC	-0.130	0.40
HLA-DR in TC	0.006	0.97
CD68 in LC	0.288	0.05
CD68 in TC	0.213	0.12
Neuropil loss in LC	0.159	0.23
Neuropil loss in TC	-0.109	0.41
No. of LC cells	0.261	0.05
DβH in LC	0.266	0.05
TH in LC	0.101	0.45
Neuromelanin	0.252	0.06
Extraneuronal NM	-0.179	0.18
% NM within LC cell	0.055	0.68

*$p<0.05$
LC Locus Coeruleus, *TC* temporal cortex

4.2.2 Alzheimer's disease pathology

The average percentage area stained by 4G8 to detect amyloid-beta and AT8 to detect p-tau was quantified as outlined in section 4.1.6 for both LC and temporal cortex sections and compared across the 3 Braak groups.

4.2.2.1 Amyloid-beta (4G8)

Comparison between the three Braak stage groups in the LC

Data were normally distributed for all three Braak groups for the LC. Due to tissue damage after bleaching the slides, 4 cases had to be excluded from the analysis even after repeating the

staining: 1 from group 0-II, 2 from group III-IV and 1 from group V-VI. There was a statistically significant difference between groups determined by one-way ANOVA $F(2,51) = 5.826$, $p=0.005$. A Tukey post hoc test revealed that the percentage protein load was statistically significantly higher in Braak group V-VI ($n=19$) than in group 0-II ($n=18$), $p=0.004$. There was no significant difference between groups 0-II and III-VI ($n=17$) or between group III-IV and V-VI.

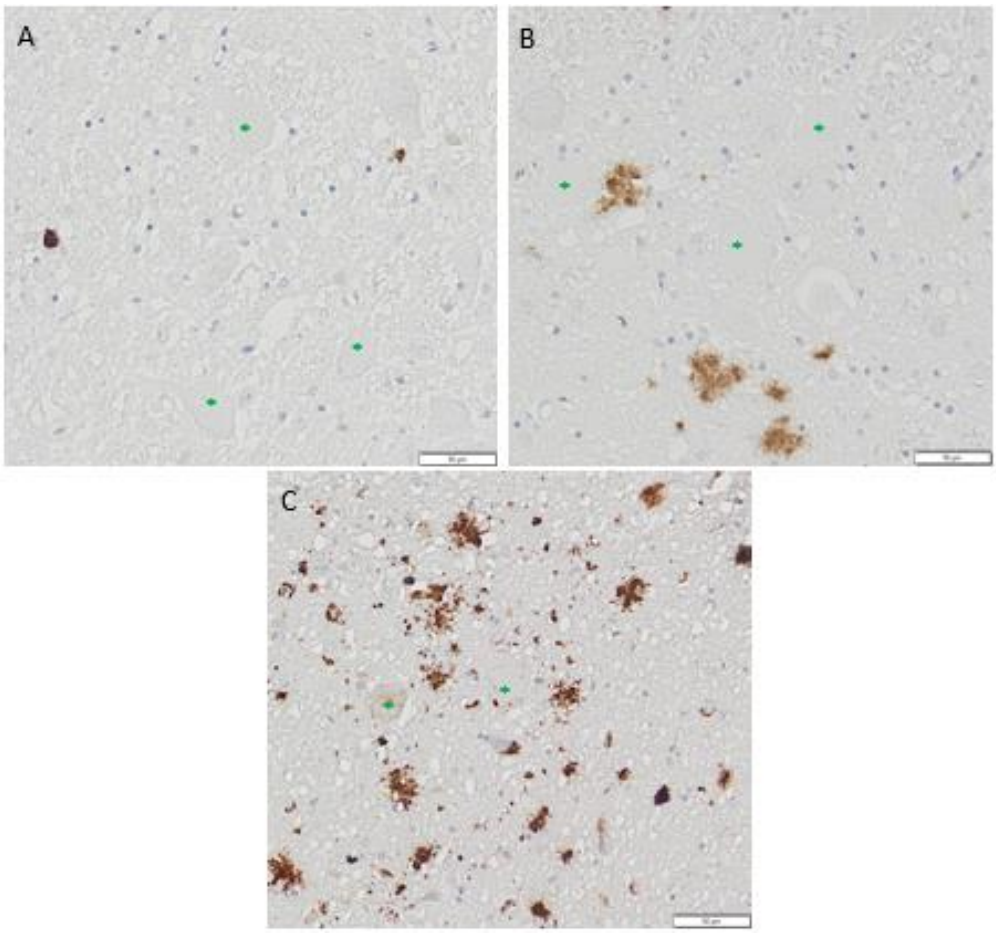


Figure 26. Illustration of the Aβ (4G8) staining in the LC between the three groups

Case 881, Braak Stage I (A), case 765, Braak stage IV (B) and case 816, Braak Stage V (C). Green asterisks denote LC cells which are only faintly visible by their cell membrane as the neuromelanin has been bleached. Haematoxylin counterstaining. Scale bar = 50μm

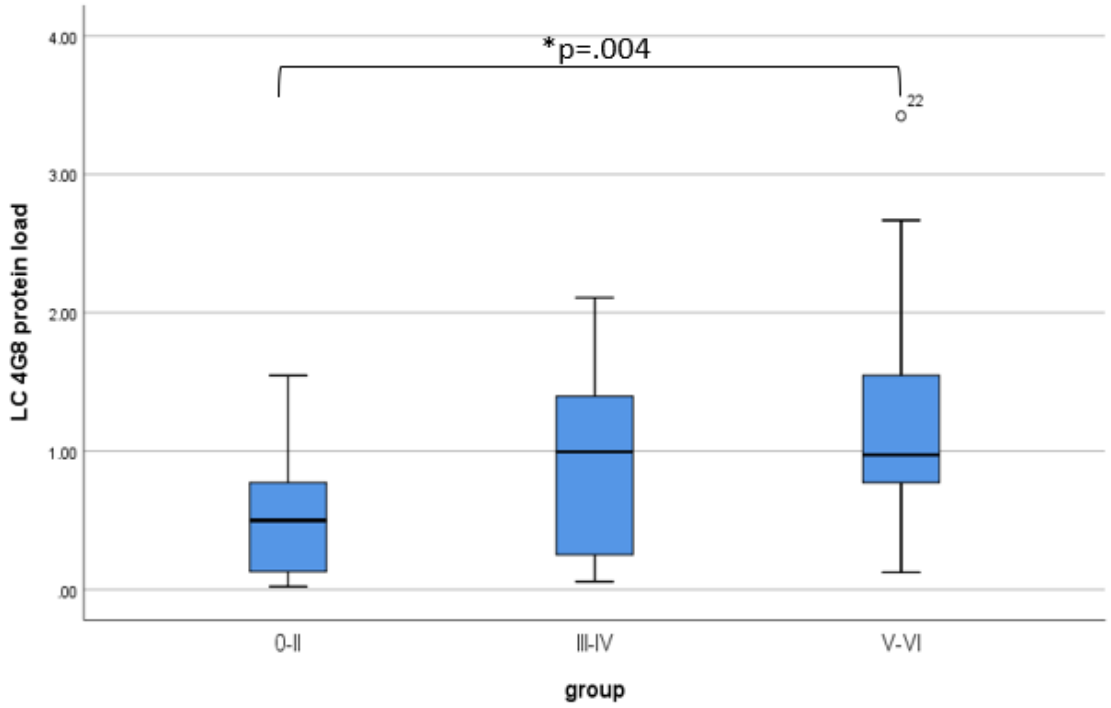


Figure 27. Aβ (4G8) protein load in the LC is significantly greater in Braak group V-VI than in group 0-II.

Comparison between the three Braak stage groups in the temporal cortex

Data were normally distributed for all three Braak groups in the temporal cortex. One-way ANOVA found the groups to be significantly different, $F(2,55) = 22.88$, $p<0.001$. A Tukey post-hoc test found significantly more staining in group III-IV (n=20) compared to group 0-II (n=18), p=0.006. and significantly more staining in group V-VI compared to group III-IV (n=20), p=0.002.

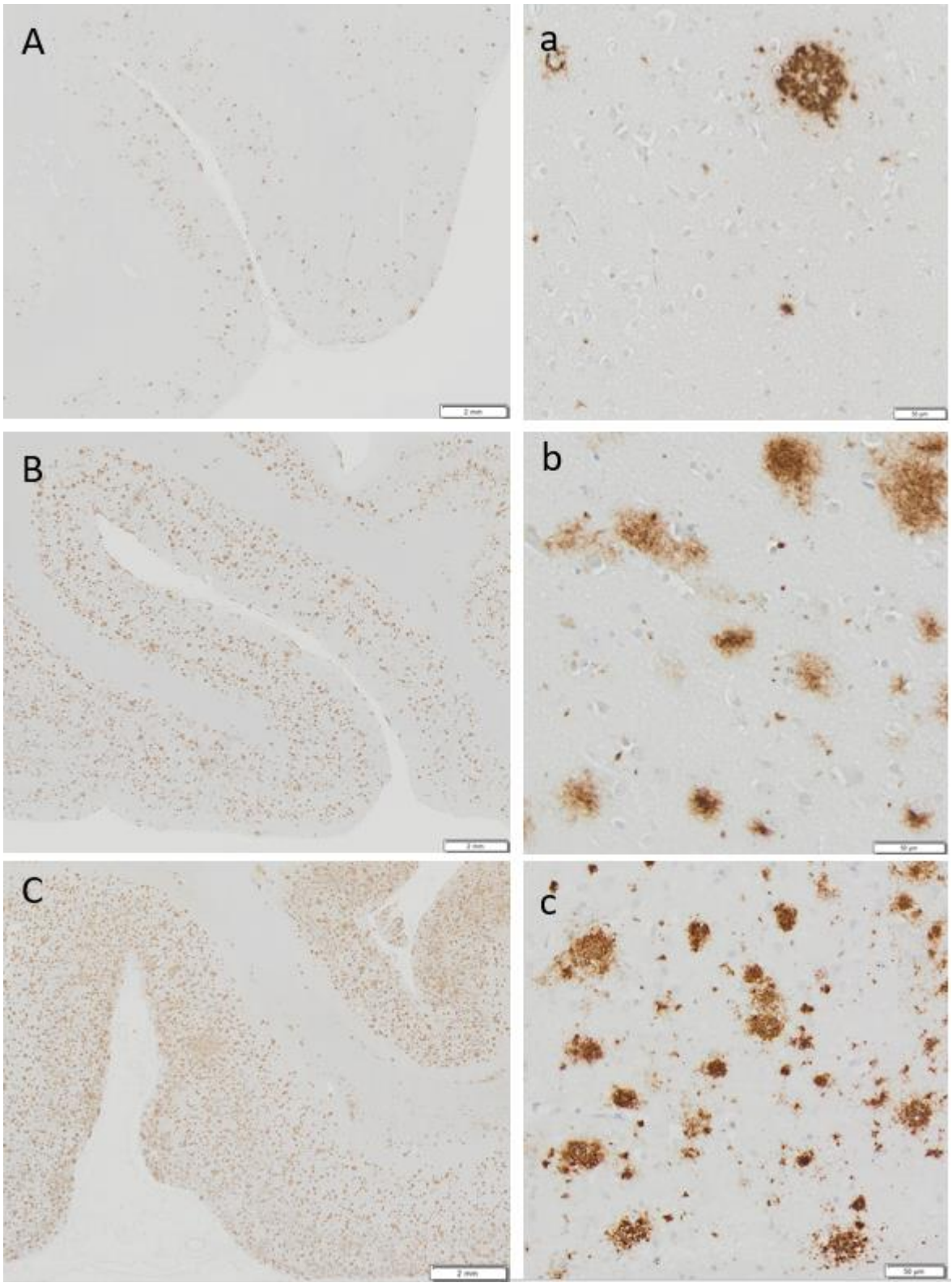


Figure 28. Illustration of Aβ (4G8) staining in the temporal cortex between the three Braak groups.

Case 766, Braak stage II (A,a), case 714, Braak stage III (B,b) and case 706, Braak stage VI (C,c). Haematoxylin counterstaining. Scale bar (A-C) = 2mm, scale bar (a-c) = 50µm.

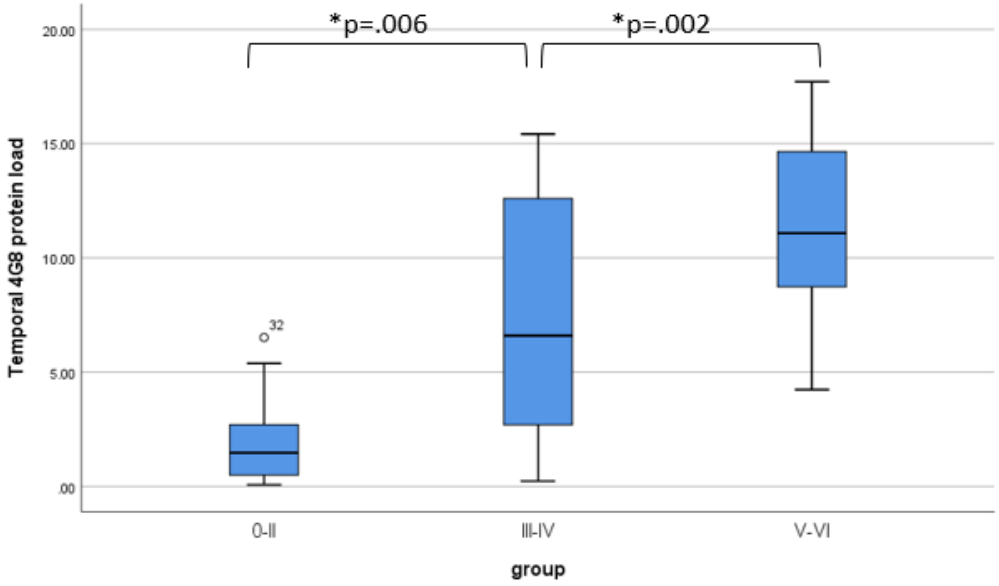


Figure 29. Aβ (4G8) protein load in the temporal cortex increases with Braak stage.

Comparison between brain areas

Aβ load in the LC correlated with Aβ load in the temporal cortex, r=.580, $p<0.001$. Paired samples t-test revealed there was significantly more Aβ in the temporal cortex compared to the LC for all Braak groups, Braak 0-II: $t(16) = -3.538$, $p=0.003$, Braak III-IV: $t(16) = -5.238$, $p<0.001$, Braak V-VI: $t(16) = -12.027$, $p<0.001$.

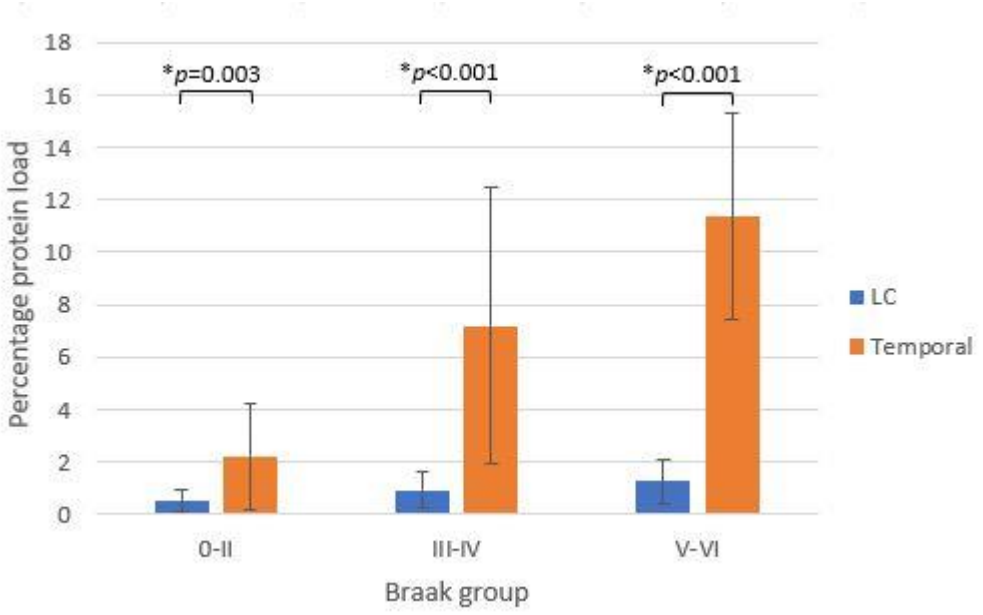


Figure 30. Comparison of Aβ (4G8) protein load in the LC and temporal cortex at each Braak group.

Error bars represent +/-1 standard deviation from mean.

4.2.2.2 P-tau (AT8)

Data were not normally distributed for either the LC or temporal cortex.

Comparison between the three Braak stage groups in the LC

Two slides had to be excluded from the analysis for the LC cases due to tissue damage, 1 from Braak group III-IV and 1 from Braak group V-VI. A Kruskal-Wallis H Test showed a statistically significant difference in AT8 staining between groups in the LC, $H(2) = 30.826$, $p<0.001$ with a mean rank score of 12.74 for Braak group 0-II (n=19), 31.11 for Braak group III-IV (n=18) and 41.79 for Braak group V-VI (n=19). Post hoc comparisons revealed that there was significantly more AT8 staining in group III-IV compared to group 0-II (p=0.002) and significantly more staining in group V-VI compared to 0-II ($p<0.001$). However there was no difference between group III-IV and V-VI.

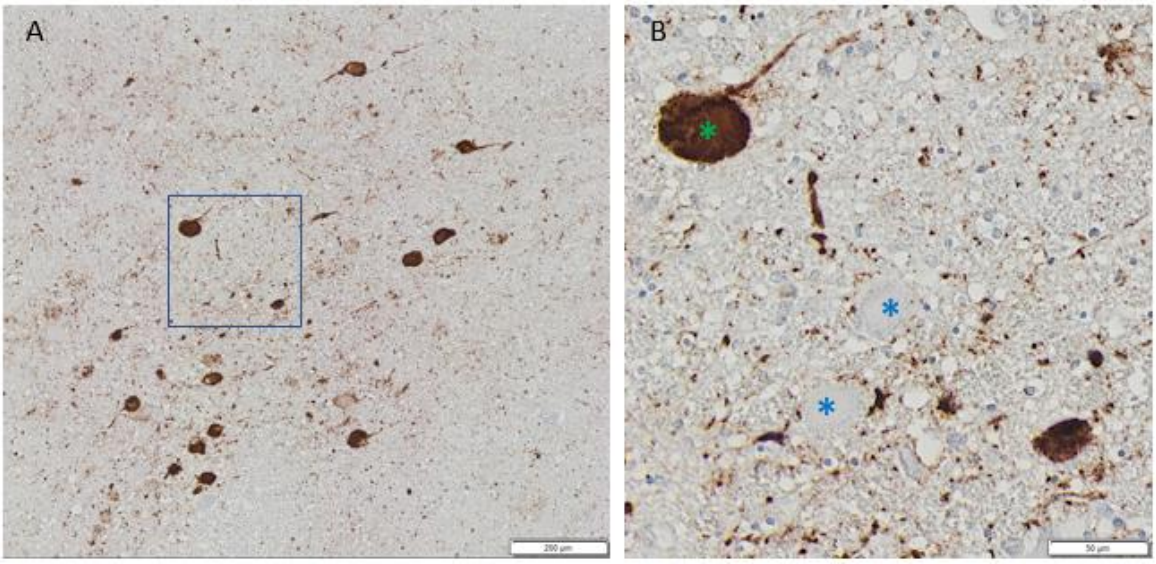


Figure 31. P-tau (AT8) staining in the LC.

Blue asterisks denote LC cells which are absent of tau pathology, whereas in some cells the entire cell body and axon are immunostained by AT8 (green asterisk). Haematoxylin counterstaining. Scale bar (A) = 200µm, (B) = 50µm.

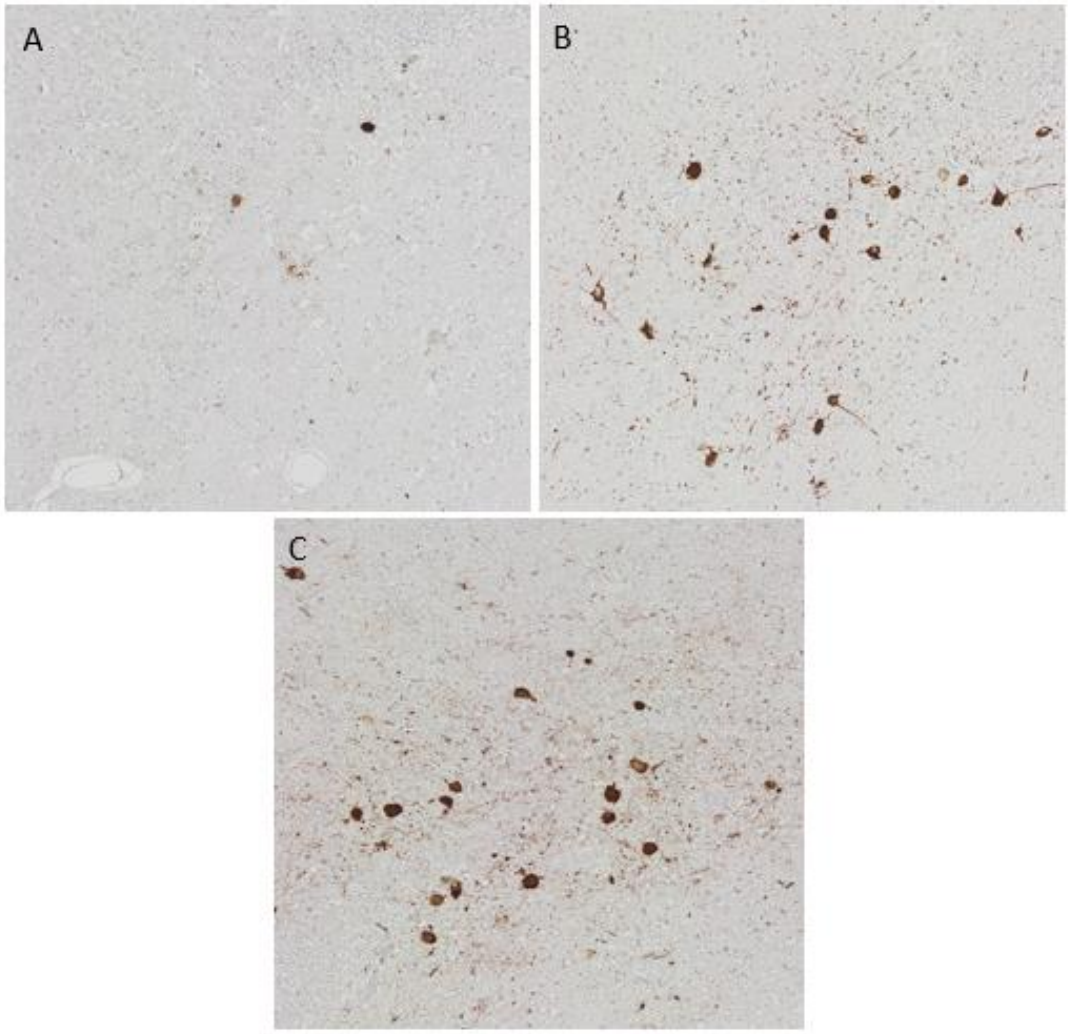


Figure 32. P-tau (AT8) staining in the LC in the three Braak groups.

Case 1082, Braak stage II (A), case 760, Braak stage IV (B) and case 717, Braak stage VI (C). Haematoxylin counterstaining.

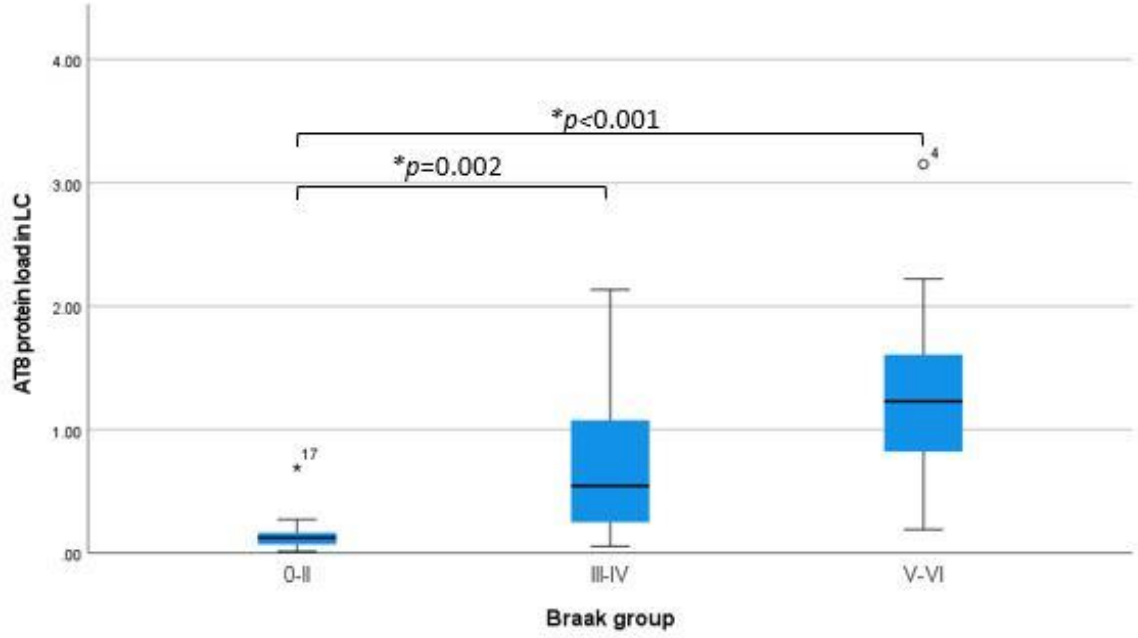


Figure 33. Protein load of p-tau (AT8) increases in the LC with Braak group.

Lines represent median scores.

Comparison between the three Braak stage groups in the temporal cortex

For the temporal cortex, 3 cases had to be excluded due to tissue damage, 2 from Braak group III-IV and 1 from Braak group V-VI. A Kruskal-Wallis H Test showed a statistically significant difference in AT8 staining between groups in the temporal cortex $H(2) = 38.834$, $p<0.001$ with a mean rank score of 12.0 for Braak group 0-II (n=19), 28.56 for Braak group III-IV (n=18) and 44.95 for Braak group V-VI (n=19). Post hoc comparisons found significantly more AT8 staining in Braak group III-IV compared to Braak group 0-II, p=0.006 (p=0.002 with age correction), and significantly more staining in Braak group V-VI compared to group III-IV, p=0.007 (p=0.008 with age correction).

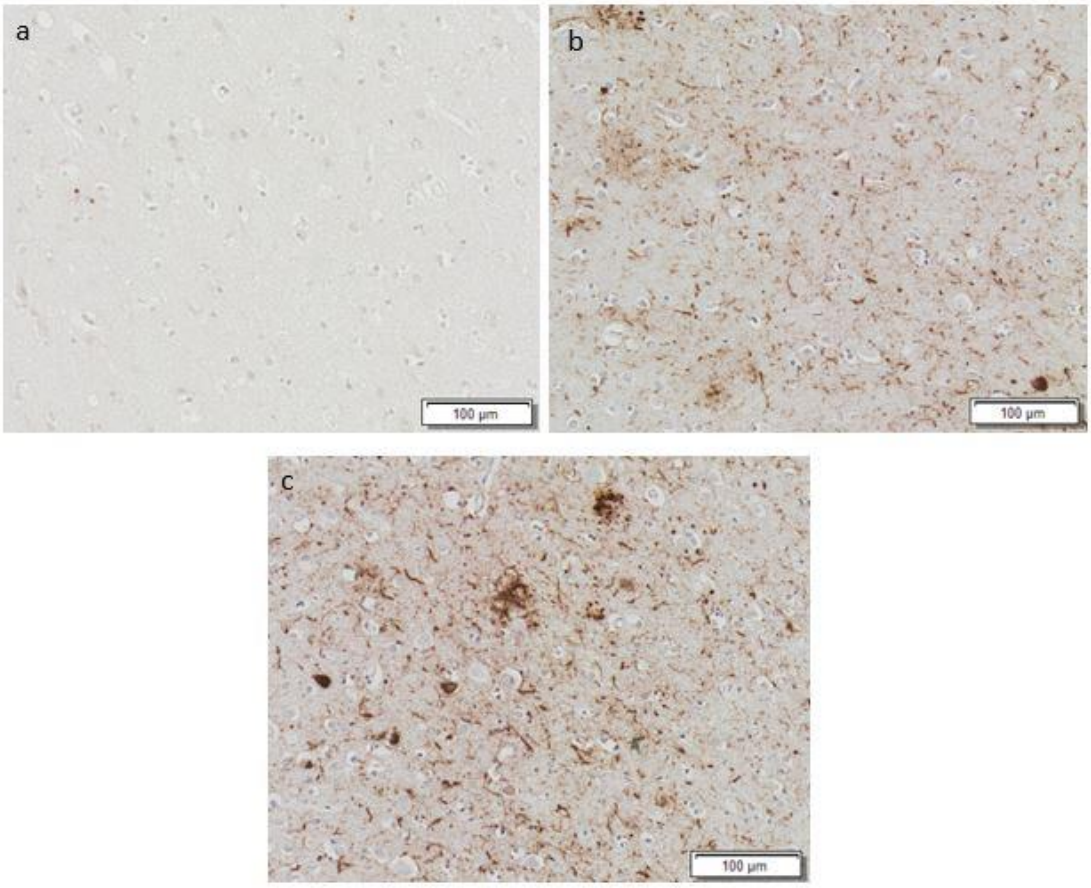


Figure 34. P-tau (AT8) staining in the temporal cortex in the three Braak groups.

Case 1068, Braak stage 0 (A), case 794, Braak stage IV (B) and case 706, Braak stage VI (C). Haematoxylin counterstaining.

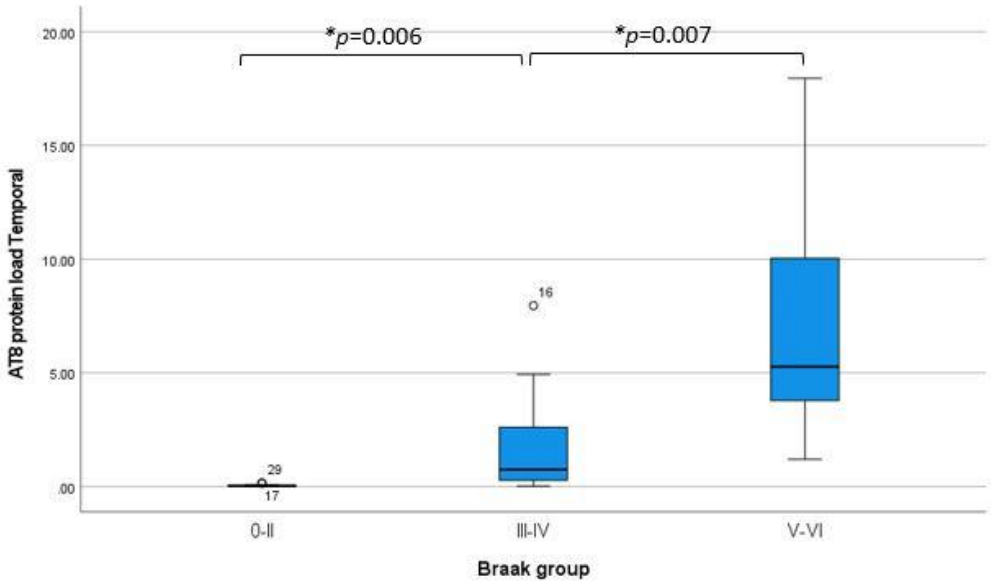


Figure 35. Percentage of p-tau (AT8) in the temporal cortex increases with Braak group.

Lines represent median scores for each group

Comparison between brain areas

P-tau load in the temporal cortex correlates with p-tau load in the LC, r_s = .694, p<0.001. Related samples test found significantly more AT8 staining in the LC compared to the temporal cortex at Braak group 0-II, t(19) = -3.783, p<0.001. However there was significantly more p-tau in the temporal cortex compared to the LC at Braak group III-IV, t(16) = 2.12, p=0.034, and Braak group V-VI, t(18) = 3.593, p<0.001.

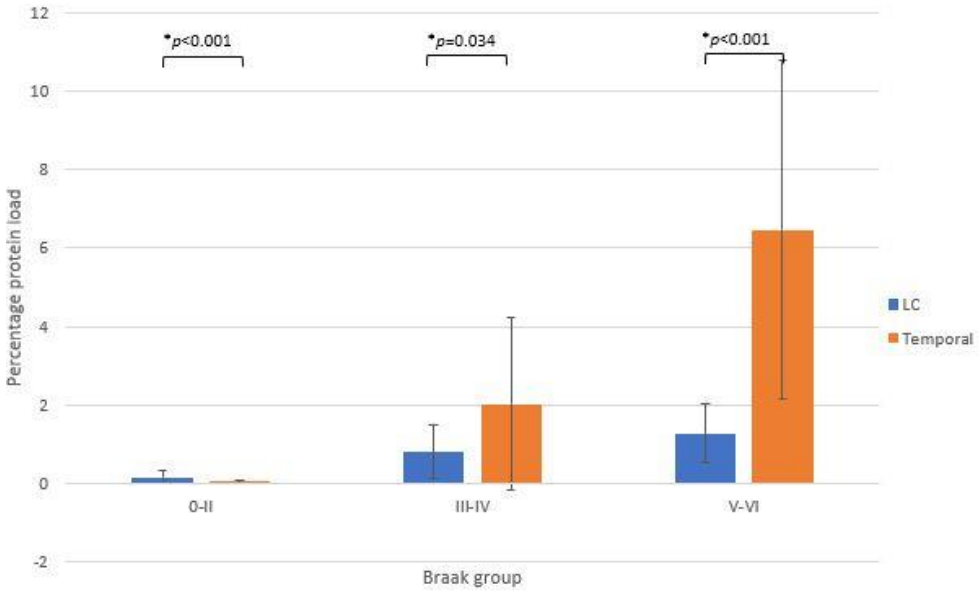


Figure 36. Comparison of p-tau (AT8) protein load in the LC and temporal cortex at each Braak group.

Error bars represent +/-1 standard deviation from mean.

4.2.2.3 Neuropil degeneration

Comparison between the three Braak stage groups in the LC

Data for neuropil degeneration in the LC were not normally distributed. For the LC, a Kruskal-Wallis test found no significant difference between Braak groups H(2) = 3.637, p=0.162.

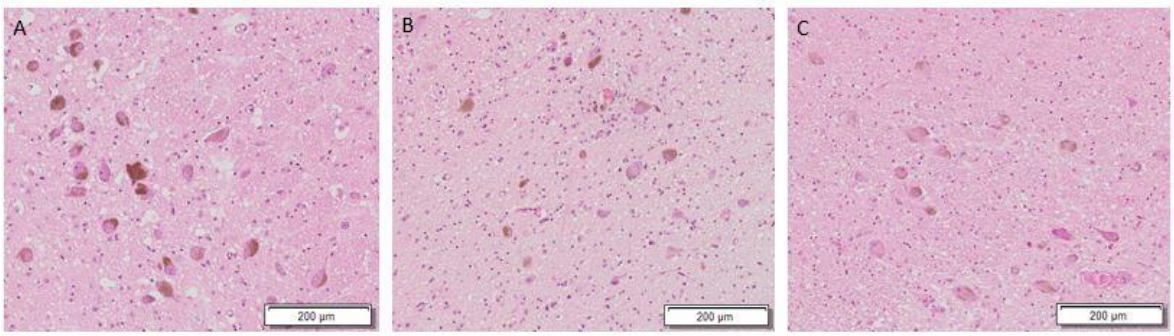


Figure 37. Illustration of haematoxylin staining depicting neuropil in the LC from the three Braak groups.

Case 1067, Braak stage I (A), case 898, Braak stage IV (B) and case 717, Braak stage VI (C).

Comparison between the three Braak stage groups in the temporal cortex

Data for neuropil degeneration in the temporal cortex were not normally distributed. For the temporal cortex, the Kruskal-Wallis test was significant, $H(2) = 7.937$, $p=0.019$ and post hoc comparisons found significantly greater neuropil degeneration at Braak group V-VI compared to group III-IV, $p=0.018$. Groups 0-II and III-IV and groups 0-II and V-VI did not differ from each other.

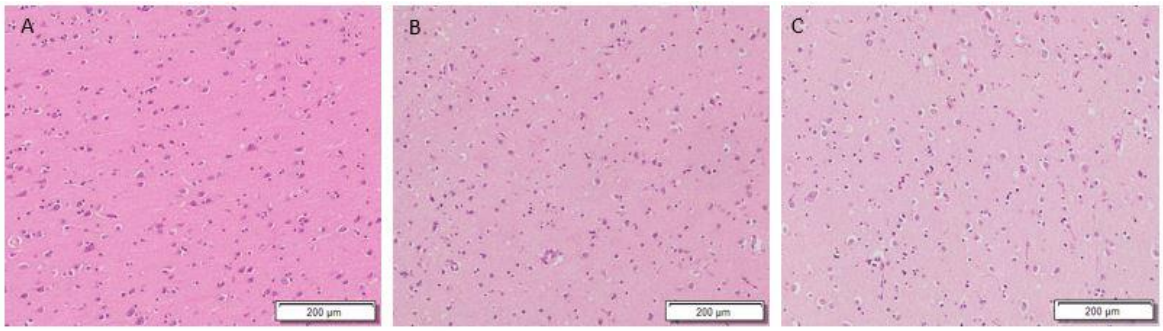


Figure 38. Illustration of haematoxylin staining depicting neuropil in the TC from the three Braak groups.

Case 1067, Braak stage I (A), case 760, Braak stage IV (B) and case 839, Braak stage VI (C).

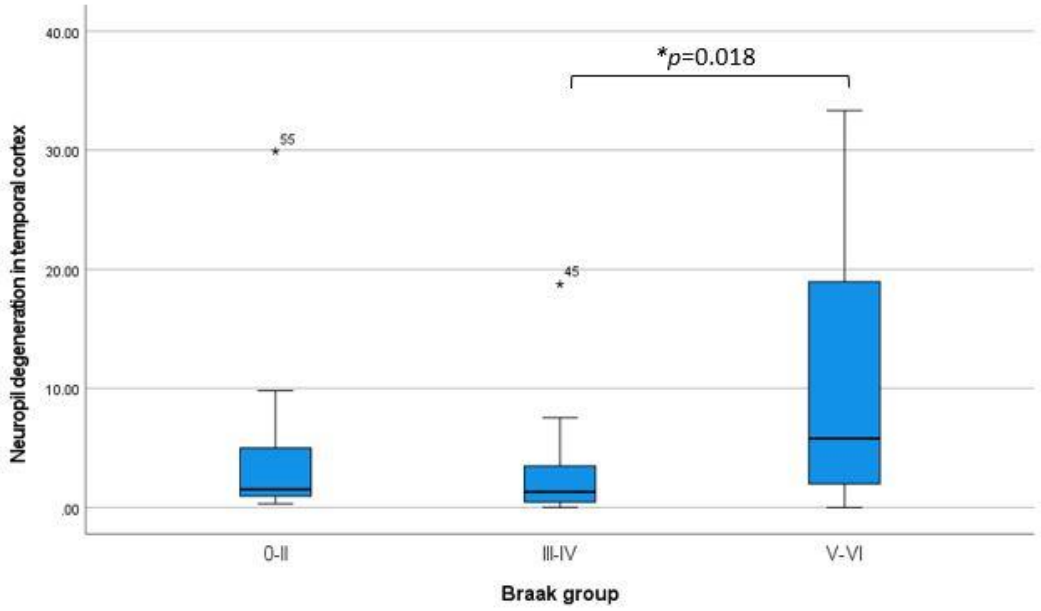


Figure 39. Neuropil degeneration in the temporal cortex was significantly greater at Braak stage V-VI compared to Braak stage III-IV.

Comparison between brain areas

Related samples t-tests found significantly greater neuropil degeneration in the LC compared to the temporal cortex at Braak group 0-II, $t(19) = 3.26$, $p=0.001$ and Braak group III-IV, $t(19) = 3.501$, $p<0.001$. However, there was no difference in the amount of neuropil degeneration between brain areas at Braak group V-VI, $p=0.627$.

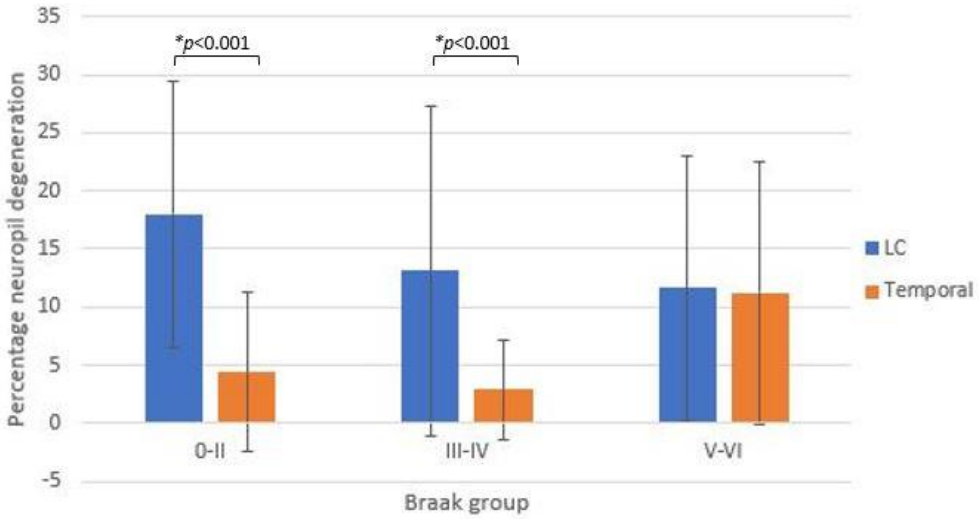


Figure 40. Comparison of neuropil degeneration in the LC and temporal cortex at each Braak group.

Error bars represent +/-1 standard deviation from mean.

4.2.3 Microglial changes

4.2.3.1 Iba1

Comparison between the three Braak stage groups in the LC

Data for the LC were normally distributed. 6 cases were excluded from the analysis due to tissue damage, 4 from Braak group 0-II and 2 from Braak group III-IV. One way ANOVA was significant, $F(2,49) = 5.806$, $p=0.005$. There was significantly more staining in Braak group V-VI ($n=20$) than Braak group 0-II ($n=15$), $p=0.004$. However there was no difference between groups 0-II and III-IV ($n=17$) or groups III-IV and V-VI.

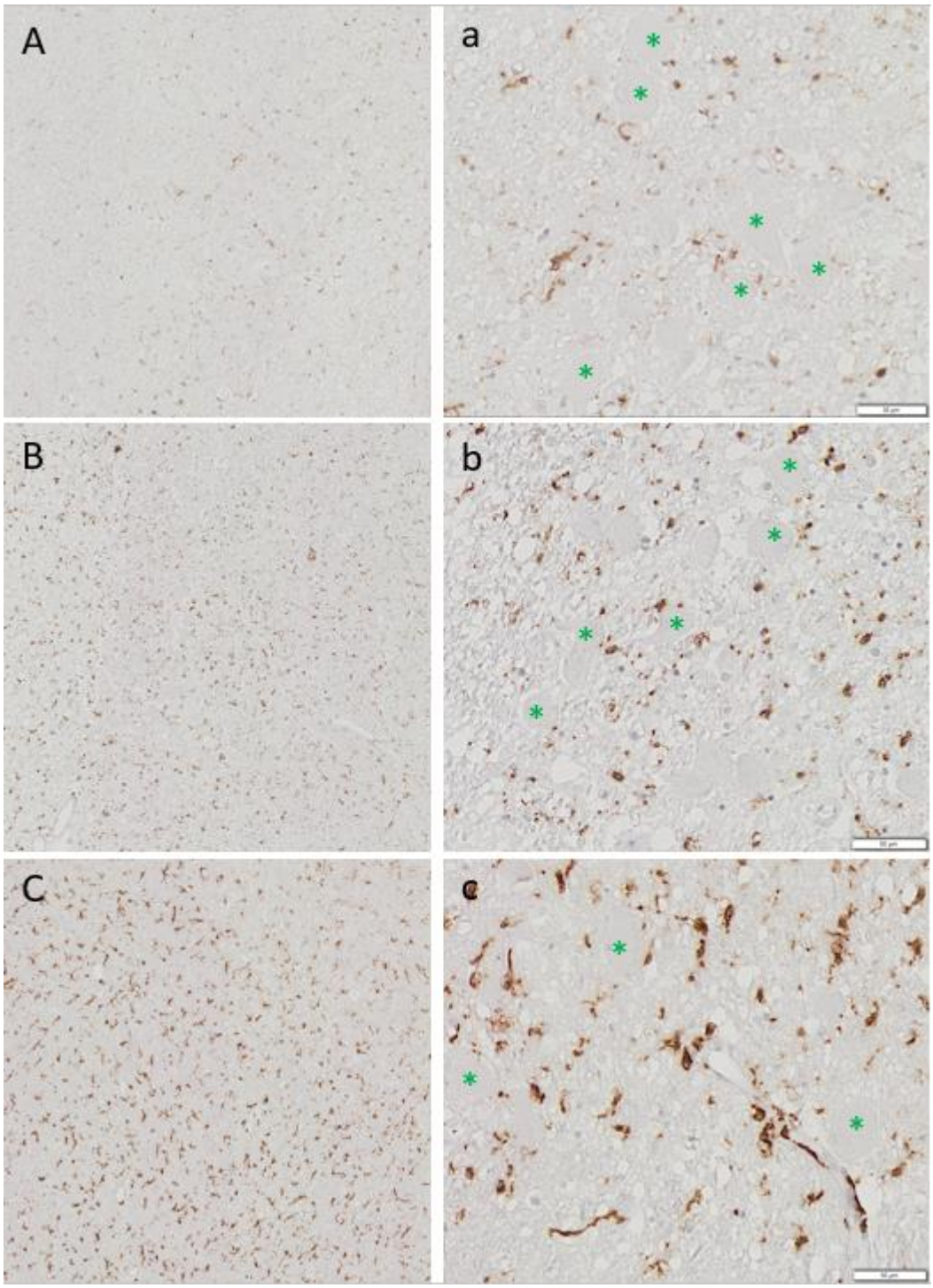


Figure 41. Illustration of Iba1 expression in the LC between the three Braak stage groups.

Case 881, Braak stage I (A,a), case 749, Braak stage III (B,b) and case 742, Braak stage VI (C,c). Haematoxylin counterstained. First column (A,B,C) shows entire LC ROI sampled, second column (a,b,c) shows magnified images, scale bar = 50µm. Green asterisks denote LC cells which are only faintly visible as the neuromelanin has been bleached.

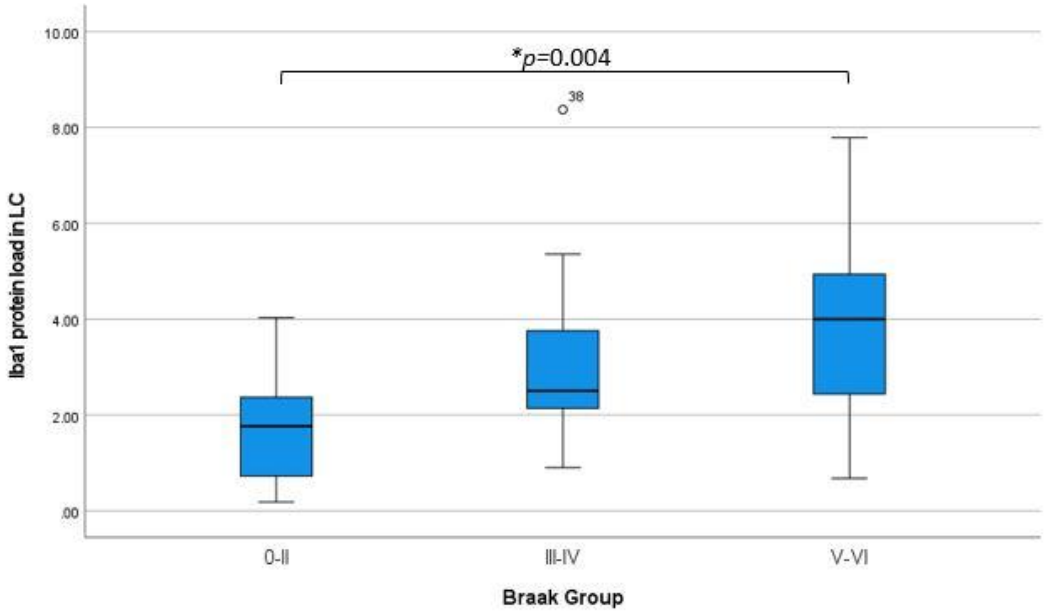


Figure 42. Percentage of Iba1 load in the LC is increased at Braak stage V-VI compared to Braak stage 0-II.

Lines represent mean values.

Comparison between the three Braak stage groups in the temporal cortex

For the temporal cortex, data were not normally distributed and Kruskal-Wallis test was not significant, $H(2) = 0.748$, $p=0.688$.

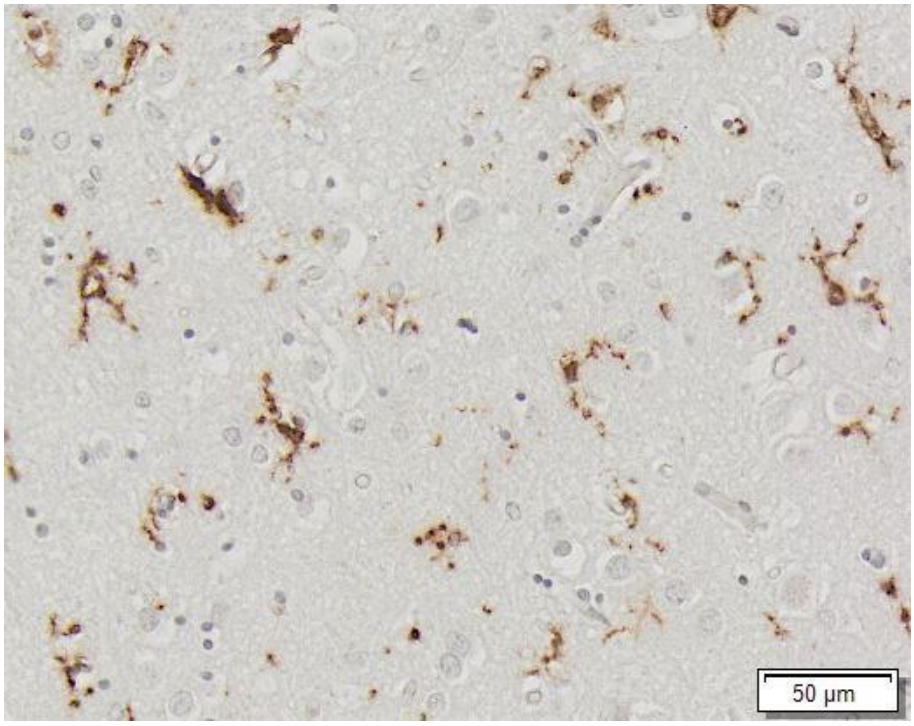


Figure 43. Illustration of Iba1 expression in the TC.

Case 763, Braak stage V. Scale bar = 50 µm.

Comparison between brain areas

At Braak stage 0-II, a paired samples t-test found no significant difference in the amount of Iba1 staining between the LC and temporal cortex, $t(14) = 1.624$, $p=0.127$. However, there was significantly more Iba1 staining in the LC compared to the temporal cortex at Braak group III-IV, $t(16) = -2.741$, $p=0.006$ and at Braak group V-VI, $t(20) = -3.808$, $p<0.001$.

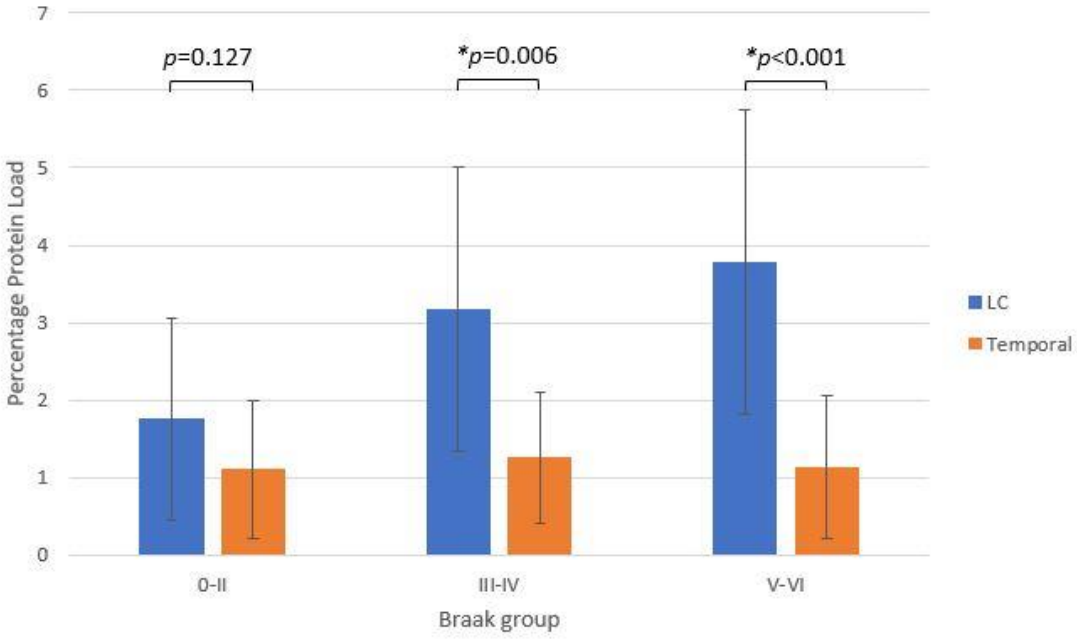


Figure 44. Comparison of Iba1 load in the LC and temporal cortex at each Braak group. Error bars represent +/-1 standard deviation from mean.

4.2.3.2 HLA-DR

Data were not normally distributed for the LC or temporal cortex. Kruskal-Wallis test found no significant difference in percentage area of HLA-DR staining in the LC across Braak groups $H(2) = 0.526$, $p=0.765$ or in the temporal cortex across Braak groups $H(2) = 0.185$, $p=0.912$. However, HLA-DR staining in the LC correlated with staining in the temporal cortex, $r_s = .521$, $p<0.001$.

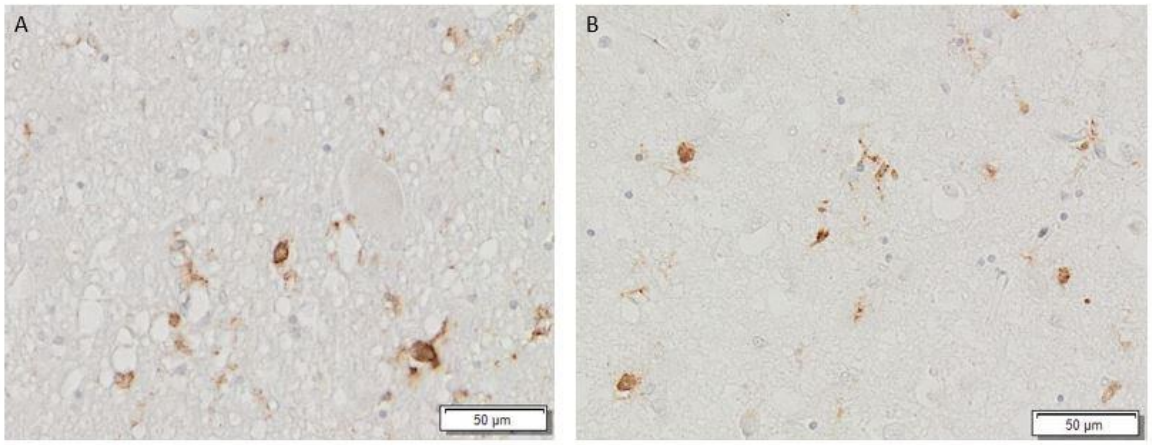


Figure 45. Illustration of HLA-DR staining in A) Locus Coeruleus and B) temporal cortex sections.

Taken from case #742, 87 year old female, Braak stage VI. Haematoxylin counterstaining, scale bar = 50µm.

4.2.3.3 CD68

Data were not normally distributed in the LC or temporal cortex. Kruskal-Wallis tests found no significant difference in the amount of CD68 staining between Braak groups in the LC sections $H(2) = 0.892$, $p=0.640$ or temporal cortex $H(2) = 1.430$, $p=0.489$. The amount of CD68 staining in the LC did not correlate with staining in the temporal cortex.

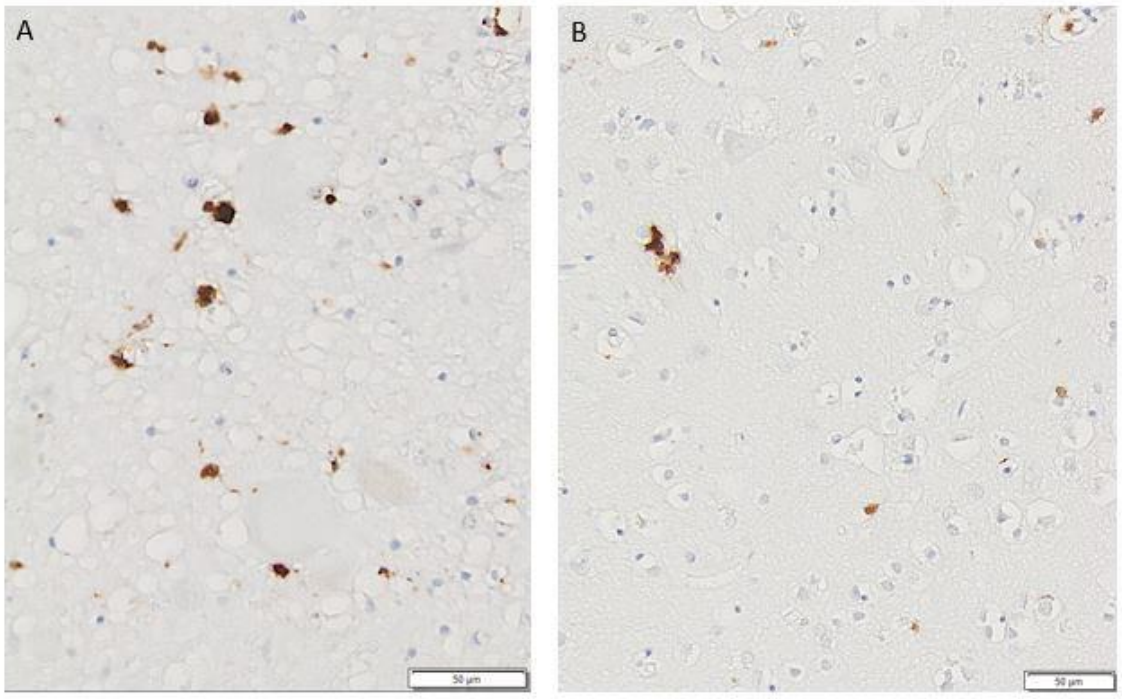


Figure 46. Illustration of CD68 staining in A) Locus Coeruleus and B) temporal cortex.

Sections take from case #706, 76 year old female, Braak stage VI. Haematoxylin counterstaining, scale bar = 50µm.

4.2.4 Locus Coeruleus cells

In the LC only, DβH expression, TH expression and neuromelanin percentage area were quantified, as well as the number of LC cells, between the three Braak groups.

4.2.4.1 DβH expression

LC sections were stained with DβH to visualise noradrenergic neurons and processes. One case from group III-IV was excluded due to tissue damage. Data for percentage area stained were not normally distributed. A Kruskal-Wallis test found a significant difference in DβH staining across Braak groups $H(2) = 12.548$, $p=0.002$. Post hoc comparisons found significantly less staining in group V-VI ($n=20$) compared to group 0-II ($n=19$), $p=0.001$. There was no difference in staining between groups 0-II and III-IV or groups III-IV and V-VI.

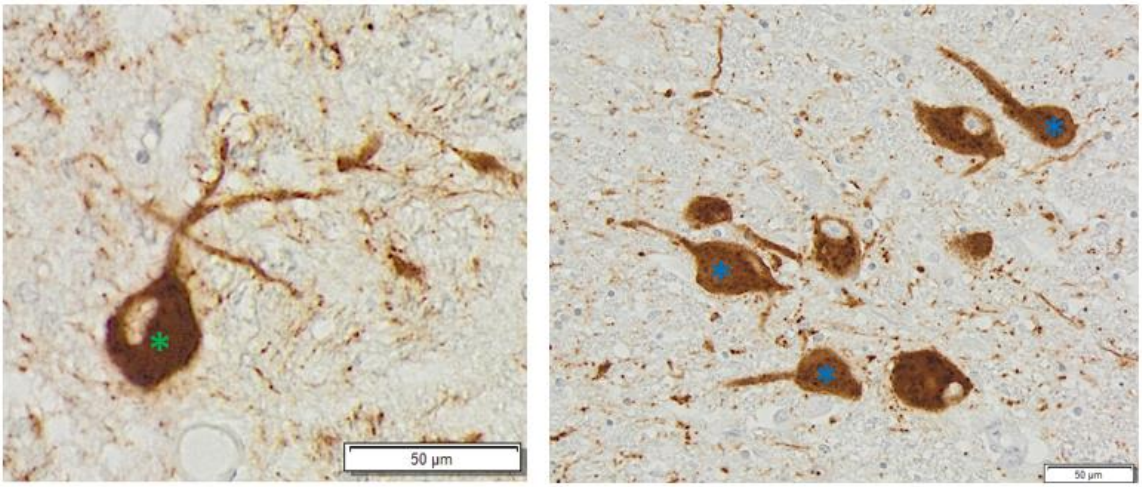


Figure 47. Examples of noradrenergic LC cells and processes stained with dopamine beta-hydroxylase.

Most LC cells have a main axon (blue asterisks) or axons which have several branches (green asterisk). Haematoxylin counterstained. Scale bar = 50µm.

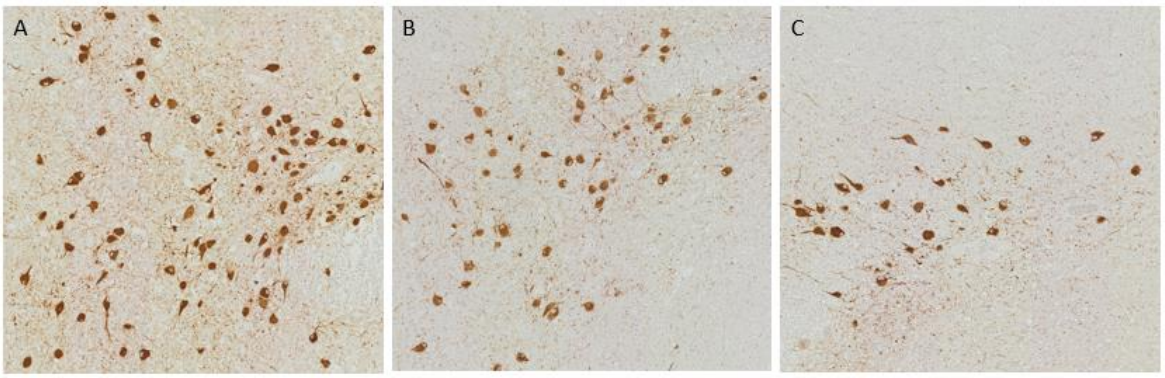


Figure 48. Illustration of Dopamine beta-hydroxylase staining of noradrenergic neurons and projections in the LC from the three Braak groups.

Case 1067, Braak stage I (A), case 760, Braak stage IV (B) and case 717, Braak stage VI (C). Haematoxylin counterstaining.

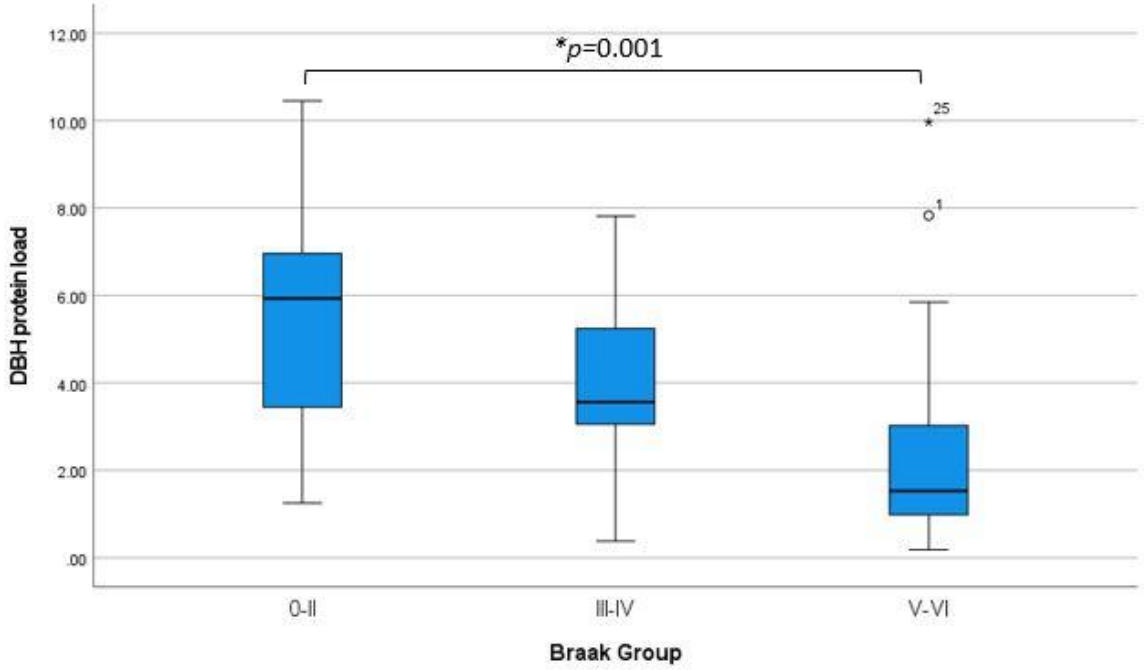


Figure 49. DβH expression in LC sections is significantly reduced in Braak group V-VI compared to Braak group 0-II.

Lines represent median values for each group.

4.2.4.2 TH expression

LC sections were also stained with tyrosine hydroxylase, a precursor for both dopamine and noradrenaline. One case from Braak group 0-II was excluded due to tissue damage. Data were normally distributed, and one way ANOVA found a significant difference in the percentage area stained, $F(2,54) = 3.39$, $p=0.041$. Post hoc comparisons found group V-VI ($n=20$) to have

significantly less staining than group 0-II (*n*=18), *p*=0.038. Group III-IV (*n*=19) did not differ significantly from the other groups.

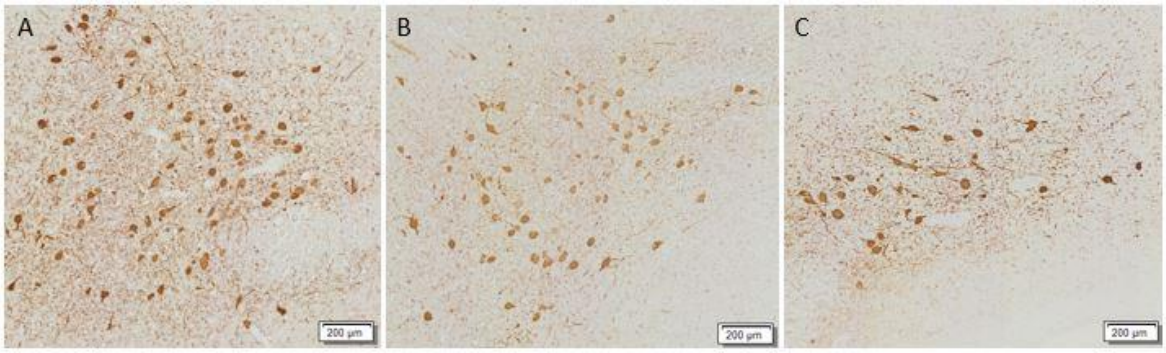


Figure 50. Illustration of tyrosine hydroxylase staining of noradrenergic neurons and projections in the LC from the three Braak groups.

Case 1067, Braak stage I (A), case 760, Braak stage IV (B) and case 717, Braak stage VI (C). Haematoxylin counterstaining, scale bar = 200µm.

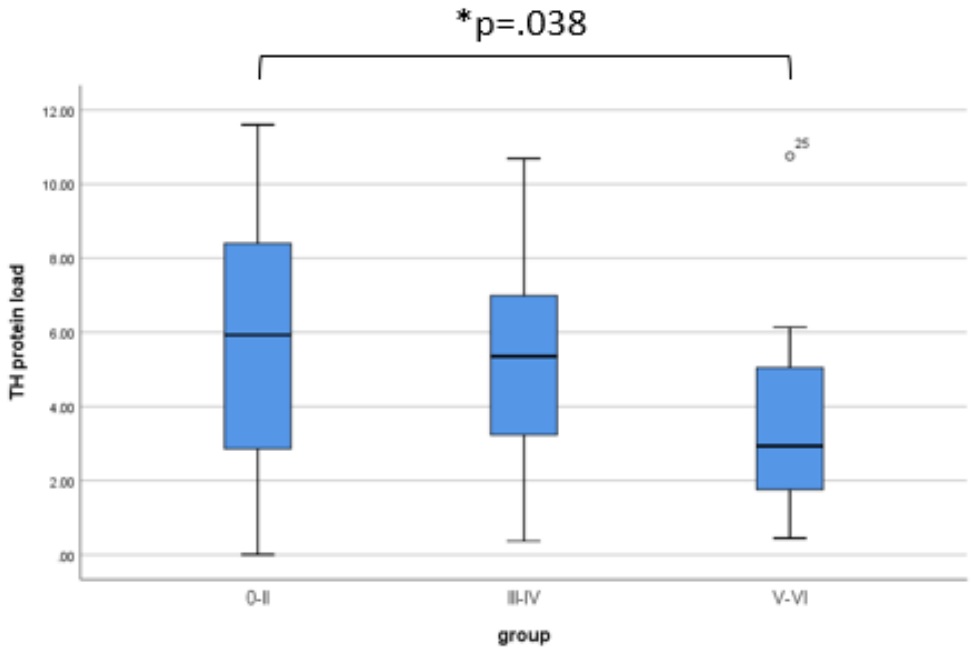


Figure 51. Tyrosine hydroxylase expression in the LC is significantly reduced in Braak group V-VI compared to group 0-II.

Lines represent median values for each group.

4.2.4.3 Number of LC cells

Data were not normally distributed, and a Kruskal-Wallis test found a significant difference between numbers of cells across Braak groups, $H(2) = 20.454$, $p<0.001$. Post hoc comparisons

found significantly fewer cells in Braak group V-VI compared to both Braak group 0-II, $p<0.001$ and Braak group III-IV, $p=0.014$. There was no difference n cell number between groups 0-II and III-IV.

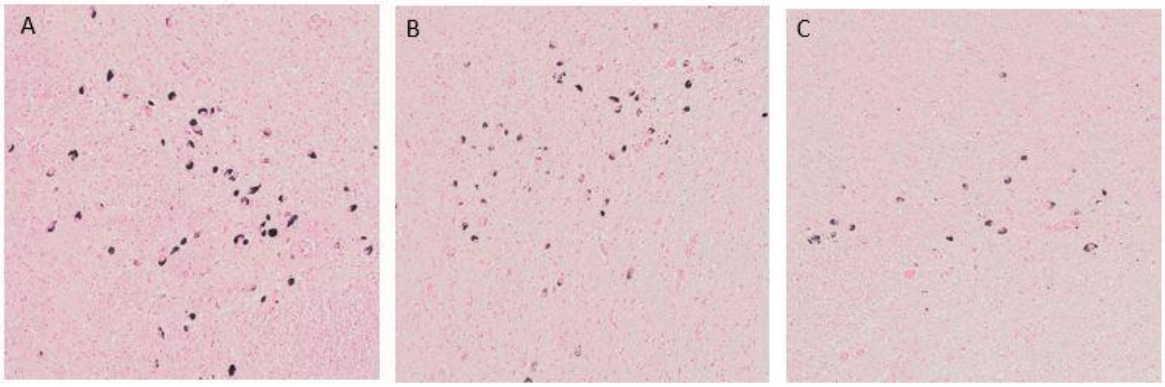


Figure 52. Neuromelanin staining of LC sections from the three Braak stage groups.

Case 1067, Braak stage I (A), Case 760, Braak stage IV (B) and Case 717, Braak stage VI (C).

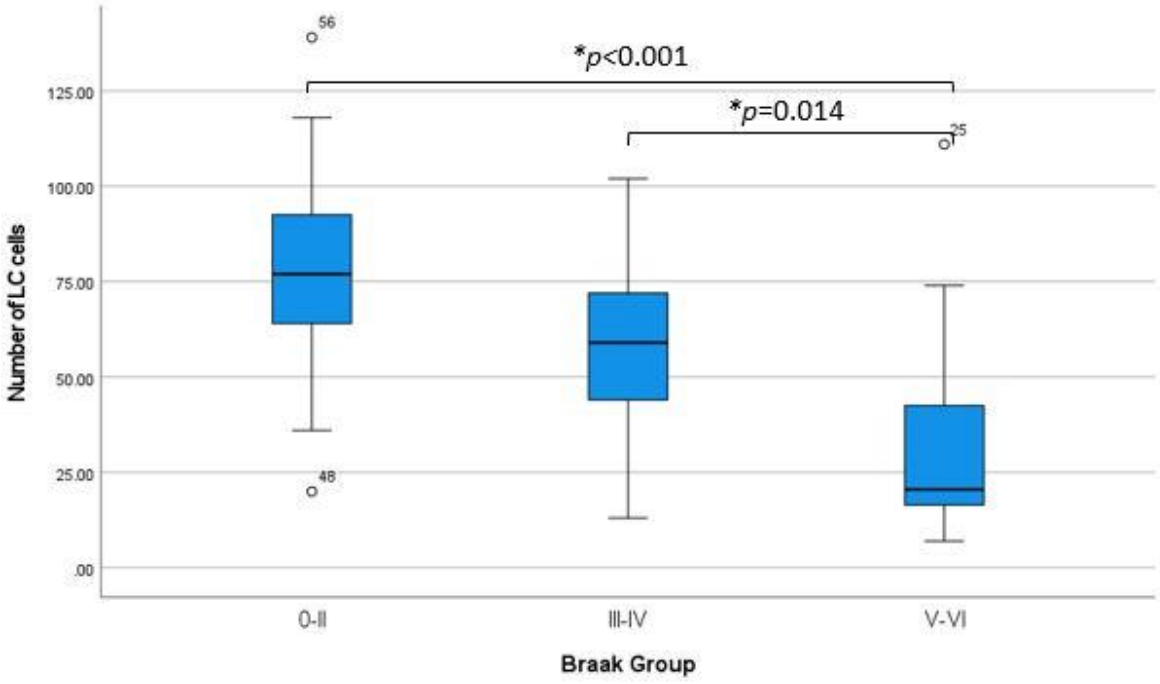


Figure 53. The number of LC cells is significantly reduced in Braak group V-VI compared to group III-IV and 0-II.

Lines represent median values for each group.

There was no significant difference in the size of the LC cells between Braak groups, $F(2,55) = 0.365$, $p=0.696$, nor was there a significant difference in shape of the LC cells as determined by their roundness across Braak groups, $F(2,55) = 0.301$, $p=0.741$.

The number of LC cells correlated with both DβH staining, i.e., the greater the number of LC cells, the greater the percentage of DβH staining observed, r_s = 0.793, p<0.001 and TH staining, r_s = 0.592, p<0.001

4.2.5 Neuromelanin

LC sections were stained for neuromelanin (NM) using the Fontana-Masson stain. The overall percentage area stained was calculated and compared across the 3 Braak groups. Data were not normally distributed and a Kruskal Wallis test found a significant difference between the Braak groups H(58) = 17.045, p<0.001. Braak groups V-VI (n=20) and III-IV (n=19) both had significantly less NM staining than group 0-II (n=19), p<0.001 and p=0.045 respectively. There was no difference in NM load between groups III-IV and V-VI.

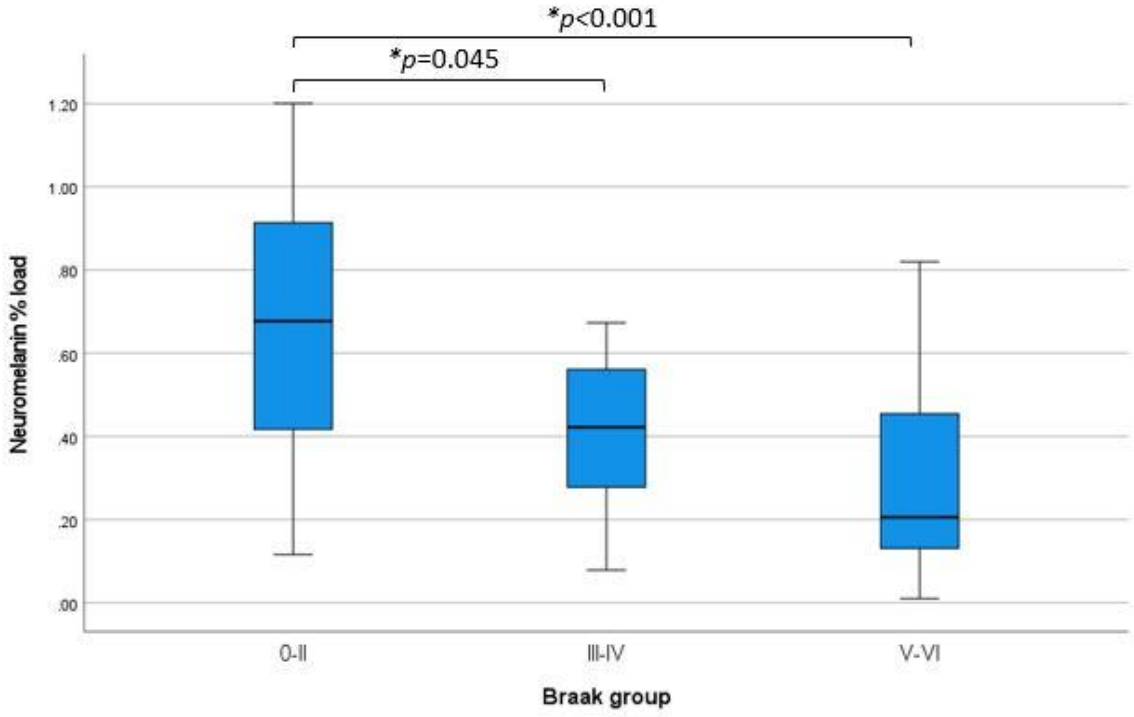


Figure 54. Overall neuromelanin protein load is significantly reduced in Braak groups III-IV and V-VI compared to Braak group 0-II

NM percentage load correlates with the number of LC cells r_s =.719, p<0.001 as well as the amount of DβH staining r_s =.658, p<0.001 and TH staining, r_s =.487, p<0.001.

The amount of NM staining within each LC cell was calculated as a percentage of the total LC cell area for each case. The average area stained for each case was compared across Braak groups. Data were normally distributed, and a one-way ANOVA showed the groups to be significantly different, F(2,55) = 3.961, p=0.025. A Tukey post-hoc test found group V-VI to have a significantly

lower average LC cell area stained with NM than group 0-II, *p*=0.029. There were no significant differences between the other groups.

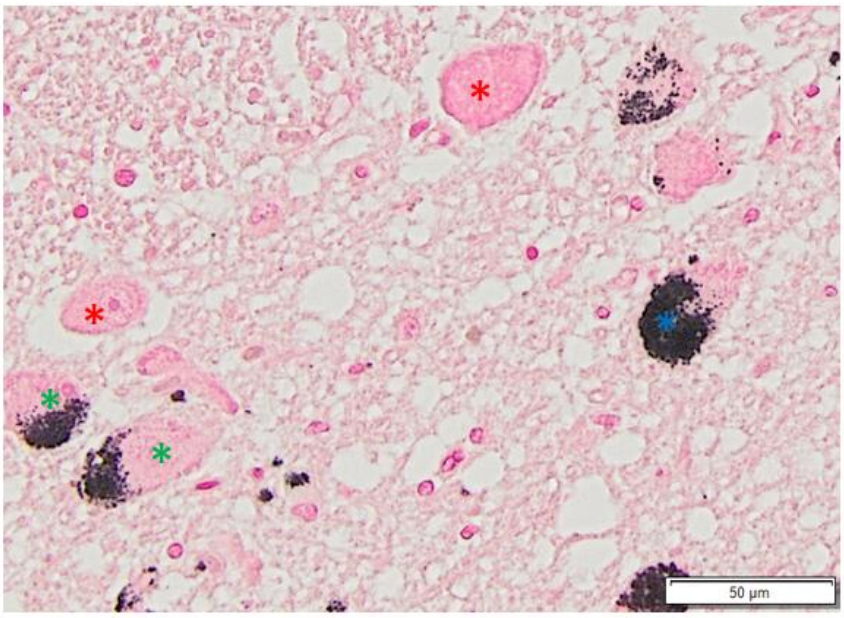


Figure 55. Illustration of LC cells which contain varying amounts of neuromelanin pigment. Some cells have complete absence of pigment (red asterisks), whilst others contain pigment accumulating at one end (green asterisks) which occurs to a greater extent in some LC cells (blue asterisks). Fontana-Masson stain with Nuclear Red counterstaining, scale bar = 50µm.

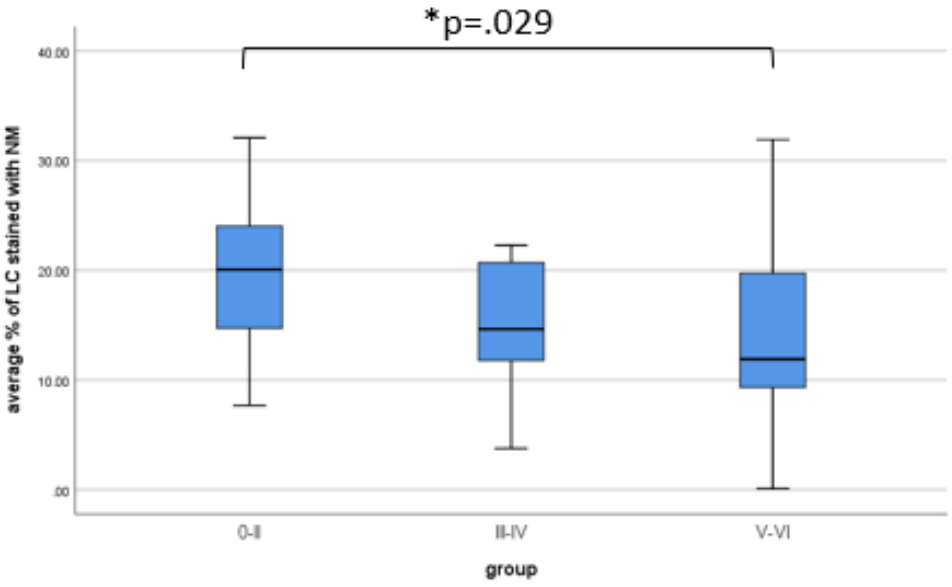


Figure 56. In Braak group V-VI LC cell bodies contained a significantly lower percentage of neuromelanin than those in Braak group 0-II.
Lines represent median values.

4.2.5.1 Extraneuronal neuromelanin

As described in the method section (4.1.6.3) extraneuronal neuromelanin was quantified using both an automated and a manual method.

Automated quantification

Data were not normally distributed among the 3 Braak groups. A Kruskal-Wallis test found a significant difference between the 3 groups $H(2) = 34.319$, $p<0.001$. Post hoc comparisons found all groups to differ significantly from each other. Group V-VI had significantly more extraneuronal NM staining than group III-IV, $p=0.027$, which in turn had significantly more staining than group 0-II, $p=0.004$.

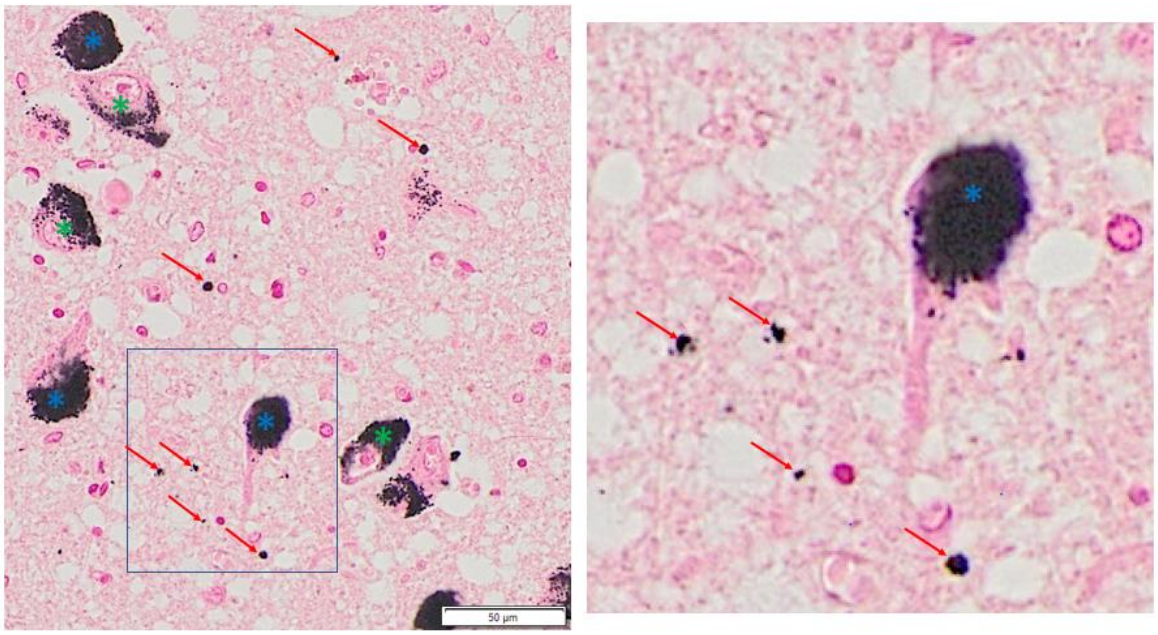


Figure 57. Illustration to show Fontana-Masson staining of neuromelanin (black) on LC section. There are multiple extraneuronal neuromelanin pigment deposits present (red arrows). Neuromelanin is found within the cell bodies of LC cells but is not present in the axons (denoted by blue asterisks), where neuromelanin does not fill the entire cell body it accumulates at one end of the cell and is absent from the nucleolus (denoted by green asterisks). Taken from case 1067, Braak stage I. Counterstained with nuclear fast red, scale bar = 50µm.

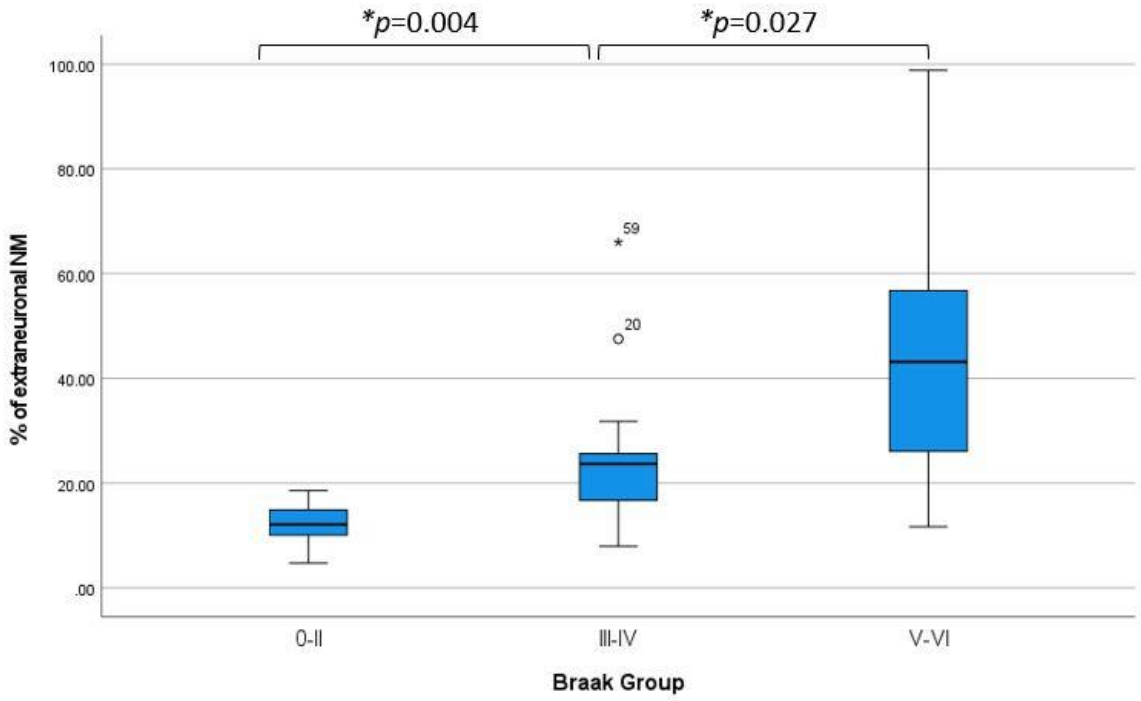


Figure 58. Extraneuronal neuromelanin deposits increase significantly with each Braak group. Lines represent median values for each group

Manual quantification

Similar results were produced as compared to the automated method. Data were not normally distributed and a Kruskal-Wallis test found a significant difference in the amount of extraneuronal NM staining between groups $H(2) = 29.792$, $p<0.001$. Similarly post hoc comparisons found group V-VI to have significantly more extraneuronal NM staining than group III-IV, $p=0.037$ and group III-IV had significantly more staining than group 0-II, $p=0.001$. This suggests that the differences observed between Braak groups was not due to NM that was counted as part of partially visible NM in LC cells, but instead due to differences in the amount of extraneuronal particles.

4.2.6 Correlations between markers

Each of the markers examined in the LC were correlated with each of the markers examined in the temporal cortex as well as with each other, to assess relationships between markers and between the two brain areas.

Braak stage was associated with greater Aβ load in the LC: $r_s = 0.457$, $p<0.001$, greater Aβ load in the temporal cortex: $r_s = 0.709$, $p<0.0001$, greater p-tau load in the LC: $r_s = 0.777$, $p<0.0001$, greater p-tau load in the temporal cortex: $r_s = 0.865$, $p<0.0001$, greater Iba1 load in the LC: $r_s = 0.467$, $p<0.001$, fewer LC cells: $r_s = -0.602$, $p<0.0001$, lower DβH load in the LC: $r_s = -0.507$,

p<0.0001, lower overall neuromelanin load in the LC: r_s = 0.553, p<0.0001 and greater extraneuronal neuromelanin load in the LC: r_s = 0.803, p<0.0001.

Greater Aβ load in the LC was associated with greater Aβ load in the temporal cortex, r_s = 0.534, p<0.0001, greater p-tau load in the temporal cortex r_s = 0.497, p<0.001 and greater p-tau load in the LC, r_s = 0.457, p<0.001. Additionally, Aβ load in the LC was associated with greater extraneuronal neuromelanin load in the LC, r_s = 0.552, p<0.0001.

Aβ load in the temporal cortex was associated with greater p-tau load in the LC, r_s = 0.566, p<0.0001 and greater p-tau load in the temporal cortex, r_s = 0.748, p<0.0001.

Greater p-tau load in the LC was associated with greater p-tau load in the temporal cortex, r_s = 0.704, p<0.0001 and with greater Aβ load in the temporal cortex, r_s = 0.566, p<0.001. Additionally, p-tau in the LC was associated with greater Iba1 load in the LC, r_s = 0.647, p<0.0001 and greater extraneuronal neuromelanin load in the LC, r_s = 0.657, p<0.0001.

Extraneuronal neuromelanin load in the LC was associated with greater Iba1 load in the LC, r_s = 0.452, p<0.001, fewer LC cells, r_s = -0.705, p<0.0001, lower DβH load in the LC, r_s = -0.567, p<0.0001 and lower overall neuromelanin load in the LC, r_s = -0.642, p<0.0001, as well as being associated with greater Aβ load in the LC, Aβ load in the temporal cortex, p-tau load in the LC, p-tau load in the temporal cortex.

Number of LC cells was associated with DβH expression, r_s = 0.793, p<0.0001, TH expression, r_s = 0.559, p<0.0001 and overall neuromelanin load, r_s = 0.719, p<0.0001. Overall neuromelanin load was also associated with DβH expression, r_s = 0.658, p<0.0001 and TH expression, r_s = 0.487, p<0.001.

HLA-DR load in the LC was associated with HLA-DR load in the temporal cortex: r_s = 0.521, p<0.001.

Figure 59, Figure 60 and Figure 61 below summarise findings from all correlations performed.

Temporal							
Locus Coeruleus	Stain	Aβ	p-tau	Iba1	HLA-DR	CD68	Neuropil loss
	Aβ	**0.534, *p*<0.0001**	**0.497, *p*=0.00018**	0.124, *p*=0.376	-0.183, *p*=0.209	-0.127, *p*=0.375	0.088, *p*=0.528
	p-tau	**0.566, *p*<0.0001**	**0.704, *p*<0.0001**	-0.070, *p*=0.611	0.079, *p*=0.582	0.019, *p*=0.893	0.149, *p*=0.272
	Iba1	0.439, *p*=0.001	0.426, *p*=0.002	0.116, *p*=0.416	-0.183, *p*=0.208	-0.073, *p*=0.619	0.104, *p*=0.462
	HLA-DR	0.029, *p*=0.855	0.015, *p*=0.925	-0.148, *p*=0.342	**0.521, *p*=0.0005**	-0.070, *p*=0.670	0.226, *p*=0.140
	CD68	0.055, *p*=0.708	0.055, *p*=0.716	0.116, *p*=0.434	-0.041, *p*=0.787	0.196, *p*=0.187	-0.130, *p*=0.372
	Neuropil Loss	-0.173, *p*=0.197	-0.320, *p*<0.05	-0.116, *p*=0.392	-0.087, *p*=0.535	0.000, *p*=0.999	-0.240, *p*=0.070
	No. of Cells	**-0.643, *p*<0.0001**	[a]**-0.677, *p*<0.0001**	-0.250, *p*=0.060	0.060, *p*=0.669	0.079, *p*=0.571	-0.254, *p*=0.054
	DβH	**-0.502, *p*<0.0001**	[b]**-0.566, *p*<0.0001**	-0.155, *p*=0.253	0.252, *p*=0.071	0.130, *p*=0.355	-0.258, *p*=0.053
	TH	**-0.437, *p*=0.00076**	**-0.514, *p*<0.0001**	-0.082, *p*=0.548	-0.067, *p*=0.636	0.059, *p*=0.670	-0.098, *p*=0.470
	% NM	**-0.493, *p*<0.0001**	**-0.612, *p*<0.0001**	-0.174, *p*=0.197	0.064, *p*=0.650	0.155, *p*=0.264	-0.267 *p*=0.043
	Ex NM	**0.724, *p*<0.0001**	**0.758, *p*<0.0001**	0.047, *p*=0.726	0.014, *p*=.0.922	-0.023, *p*=0.867	0.285 *p*=0.030

Significant correlations with *p*-value ≤0.00076 (Bonferroni corrected for multiple comparisons) in bold.
[a] adjusted correlation -0.61, p<0.0001 correcting for age and gender
[b] adjusted correlation -0.54, p<0.0001 correcting for age and gender

Figure 59. Significant Spearman's Rho (r_s) correlations between markers in LC and temporal cortex

Temporal							
Temporal	Stain	Aβ	p-tau	Iba1	HLA-DR	CD68	Neuropil Loss
	Braak stage	**0.709, *p*<0.0001**	**0.865, *p*<0.0001**	-0.007, *p*=0.956	-0.001, *p*=0.993	-0.056, *p*=0.685	0.296, *p*=0.023
	p-tau	**0.748, *p*<0.0001**					
	Iba1	0.031, *p*=0.820	0.101, *p*=0.465				
	HLA-DR	0.029, *p*=0.838	0.100, *p*=0.483	0.098, *p*=0.484			
	CD68	-0.185, *p*=0.177	-0.148, *p*=0.296	0.181, *p*=0.191	0.116, *p*=0.414		
	Neuropil loss	0.059, *p*=0.662	0.210, *p*=0.120	-0.267, *p*=0.043	-0.150, *p*=0.278	-0.077, *p*=0.576	

Significant correlations with *p*-value ≤0.0024 (Bonferroni corrected for multiple comparisons) in bold.

Figure 60. Significant Spearman's Rho (r_s) correlations between markers in the temporal cortex.

	Locus coeruleus											
Locus Coeruleus	Stain	Aβ	p-tau	Iba1	HLA-DR	CD68	Neuropil Loss	No. of cells	DβH	TH	%NM	Ex NM
	Braak stage	**0.457, *p*=0.00052**	**0.777, *p*<0.0001**	**0.467, *p*=0.00059**	0.065, *p*=0.676	-0.089, *p*=0.544	-0.209, *p*=0.115	**-0.602, *p*<0.0001**	**-0.507, *p*<0.0001**	-0.352, *p*=0.007	**-0.553, *p*<0.0001**	**0.803, *p*<0.0001**
	p-tau	**0.457, *p*=0.00066**										
	Iba1	0.409, *p*=0.004	**0.647 *p*<0.0001**									
	HLA-DR	-0.096, *p*=0.550	0.163, *p*=0.301	-0.070, *p*=0.664								
	CD68	-0.049, *p*=0.744	0.023, *p*=0.878	-0.022, *p*=0.886	0.078, *p*=0.643							
	Neuropil Loss	-0.181, *p*=0.189	-0.130, *p*=0.338	-0.144, *p*=0.307	0.075, *p*=0.626	-0.136, *p*=0.353						
	No. of Cells	-0.441, *p*=0.00085	-0.296, *p*=0.027	-0.183, *p*=0.193	0.061, *p*=0.694	-0.244, *p*=0.091	0.244, *p*=0.065					
	DβH	-0.332, *p*=0.15	-0.211, *p*=0.121	-0.121, *p*=0.398	0.106, *p*=0.492	-0.064, *p*=0.668	0.099, *p*=0.463	***0.793, *p*<0.0001**				
	TH	-0.366, *p*=0.007	-0.241, *p*=0.076	-0.160, *p*=0.257	0.084, *p*=0.593	-0.212, *p*=0.144	0.063, *p*=0.640	**0.592, p<0.0001**	**0.559, p<0.0001**			
	% NM	-0.358, *p*=0.008	-0.311, *p*=0.020	-0.134, *p*=0.343	0.024, *p*=0.876	0.005, *p*=0.970	0.193, *p*=0.146	**0.719, *p*<0.0001**	**0.658, *p*<0.0001**	**0.487, p=0.00012**		
	Ex NM	**0.552, *p*<0.0001**	**0.657, *p*<0.0001**	**0.453, *p*=0.00074**	0.110, *p*=0.476	0.058, *p*=0.690	-0.266, *p*=0.044	**-0.705, *p*<0.0001**	**-0.567, *p*<0.0001**	-0.390, p=0.003	**-0.642, *p*<0.0001**	

Significant correlations with *p*-value ≤0.00076 (Bonferroni corrected for multiple comparisons) in bold.
* adjusted correlation 0.78, p<0.0001 correcting for age and gender

Figure 61. Significant Spearman's Rho (r_s) correlations between markers in the LC.

4.2.7 Summary

A summary of the data including either means and standard deviations or medians and interquartile ranges are displayed in Table 16. In addition the data are schematically reported below Figure 62.

Table 16. Summary of protein loads for each marker in each brain area (LC or temporal cortex) for each Braak group

Marker	**Braak 0-II**	**Braak III-IV**	**Braak V-VI**	***p* value**
Aβ (4G8) in LC (%)	0.52 ±0.82	0.92 ±0.68	1.26 ±.82	0.005*
Aβ (4G8) in TC (%)	2.21 ±2.21	7.2 ±5.29	11.36 ±3.91	<0.001*
p-tau (AT8) in LC (%)	0.12 [0.06–0.17]	0.55 [0.25-1.08]	1.22 [0.80–1.74]	<0.001*
p-tau (AT8) in TC (%)	0.04 [0.01-0.06]	0.75 [0.24-2.76]	6.80 [3.79-10.1]	<0.001*
Neuropil degeneration in LC (%)	16.69 [11.13-26.43]	6.26 [1.03-19.08]	8.26 [1.67 – 16.0]	0.162
Neuropil degeneration in TC (%)	1.53 [0.9-5.37]	1.32 [0.42-3.95]	5.80 [1.92-19.4]	0.019*
Iba1 in LC (%)	1.76 ±1.30	3.09 ±1.82	3.78 ±1.97	0.005*
Iba1 in TC (%)	0.80 [0.60-1.17]	1.10 [0.66-1.84]	0.89 [0.41-1.67]	0.688
HLA-DR in LC (%)	0.08 [0.13-0.39]	0.10 [0.18-0.45]	0.09 [0.01-0.24]	0.765
HLA-DR in TC (%)	0.06 [0.01-0.18]	0.04 [0.01-0.15]	0.05 [0.0-0.11]	0.912
CD68 in LC (%)	0.11 [0.06- 0.36]	0.14 [0.05-0.23]	0.08 [0.04-0.19]	0.640
CD68 in TC (%)	0.06 [0.04-0.11]	0.05 [0.02-0.15]	0.06 [0.01-0.14]	0.489
DβH in LC (%)	5.93 [3.24-7.41]	3.56 [3.03-5.29]	1.53 [0.92-3.19]	0.002*
TH in LC (%)	5.94 ±3.52	5.19 ±2.82	3.51 ±2.50	0.041*
No. of LC cells	77 [36-96]	59 [42-74]	20.5 [16.25-42.75]	<0.001*
Neuromelanin (%)	0.68 [0.42-1.01]	0.42 [0.26-0.56]	0.21 [0.13-0.46]	<0.001*
Extraneuronal NM (%)	12.11 [9.49-14.89]	23.67 [16.07-26.25]	43.16 [25.99-57.0]	<0.001*
% NM within LC cell	20.47 ±7.0	15.54 ±5.25	14.58 ±8.21	0.025*

*$p<0.05$

p values calculated using either ANOVA or Kruskal-Wallis test

Data presented as either mean ±standard deviation for normally distributed data or median [Interquartile range] for non-parametric data

LC Locus Coeruleus, *TC* temporal cortex

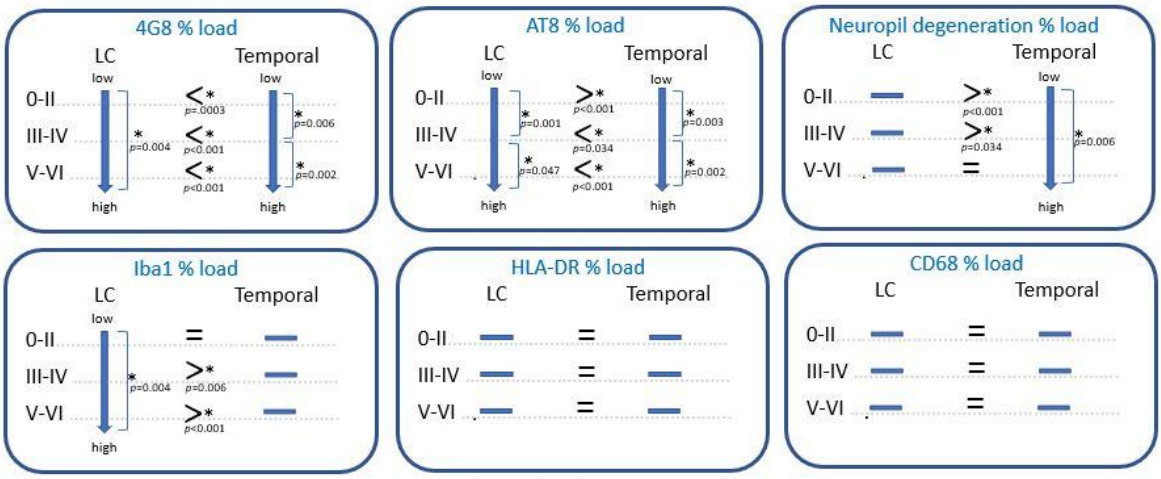


Figure 62. Schematic diagram to summarise findings within each brain area and Braak stage group, with comparison between the LC and temporal cortex within the same Braak stage group.

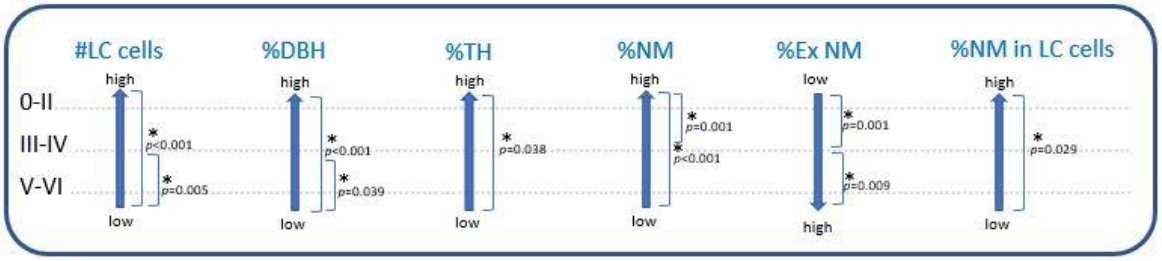


Figure 63. Schematic diagram to summarise the neuronal and NM findings in the LC for each Braak stage group

4.3 Results – Mesoscale Discovery Assay

Comparison between the three Braak stage groups in the LC

In the LC samples, of the 30 analytes examined, a Kruskal-Wallis test found a significant difference between Braak groups for 7 of the markers. All other analytes examined were not significantly different between Braak groups.

There was a significant difference in

- MCP-4 between Braak groups, $H(2) = 6.534$, $p=0.038$. (However this was not significant after correction for age and gender, $p=0.055$). Post hoc tests revealed that the trend significance was in decreased MCP-4 in Braak group V-VI compared to 0-II, $p=0.013$

(p=0.047 after correction for age and gender). There was no significant difference between groups 0-II and III-IV or III-IV and V-VI.

- MIP-1β between Braak groups, $H(2) = 10.086$, p=0.006 (p=0.05 after correction for age and gender) with significant decrease in MIP-1β in Braak group V-VI compared to Braak group 0-II, p=0.003 (p<0.001 after age and gender correction) and III-IV, p=0.009 (p=0.044 after age and gender correction). There was no difference between groups 0-II and III-IV.

- IL-8(HA) between Braak groups, $H(2) = 6.165$, p=0.046 (p=0.044 after correction for age and gender) with significant decrease in IL-8(HA) in Braak group V-VI compared to Braak group 0-II, p=0.022 (p=0.003 after correction for age and gender) and III-IV, p=0.039 (p=0.02 after correction for age and gender). There was no difference between groups 0-II and III-IV.

- TARC between Braak groups, $H(2) = 7.277$, p=0.026 (p=0.028 after correction for age and gender) with significant decrease in Braak group V-VI compared to Braak group 0-II, p=0.016 (p=0.006 after correction for age and gender) and III-IV, p=0.020 (p=0.049 after correction for age and gender). There was no difference between groups 0-II and III-IV.

- MDC between Braak groups, $H(2) = 7.206$, p=0.027 (p=0.002 after correction for age and gender) with significant decrease in Braak group V-VI compared to Braak group 0-II, p=0.007 (p=0.002 after correction for age and gender). There was no difference between groups 0-II and III-IV or groups III-IV and V-VI.

- GM-CSF between Braak groups, $H(2) = 6.565$, p=0.038 (but not significant after correction for age and gender, p=0.066). Post hoc tests revealed that the trend significance was in the decrease in Braak group V-VI compared to Braak group III-IV, p=0.011 (p<0.001 after correction for age and gender). There was no difference between groups 0-II and III-IV or groups 0-II and III-IV.

- IL-17A between Braak groups, $H(2) = 6.585$, p=0.037 (but not significant after correction for age and gender, p=0.066). Post hoc tests revelated that the trend significance was in the decrease in Braak group V-VI compared to Braak group 0-II, p=0.011 (p=0.08 after correction for age and gender). There was no difference between groups 0-II and III-IV or groups III-IV and V-VI.

Comparison between the three Braak stage groups in the temporal cortex

In the temporal cortex samples, of the 30 analytes examined, Kruskal-Wallis test found a significant difference between Braak groups for only one of the markers, IL-15, $H(2) = 7.461$, $p=0.024$. Post hoc comparisons found there to be a significant increase in IL-15 in group V-VI compared to group 0-II (Mann Whitney, $H(1) = 221.0$, $p=0.006$) which remained significant after correcting for age and gender ($p=0.03$). There was no significant difference between groups 0-II and III-IV or III-IV and V-VI.

All other analytes examined were not significantly different between Braak groups.

The data for all analytes for both LC and the temporal cortex is provided in Appendix I.

4.4 Discussion

Whilst several studies have demonstrated that the LC undergoes significant degeneration in AD [113,132,194,197] and more recently that p-tau accumulates first in the axons of the LC and then in the LC cell bodies before being detected cortically [214], inflammatory changes associated with these changes within the LC have surprisingly not been explored. It was hypothesised that inflammatory changes in the LC would occur early in the disease process and would be associated with LC neuronal loss. It was thought there would be a temporal relationship between changes in the LC and changes in LC cortical projection areas, in this study the middle superior temporal gyrus. It was hypothesised that as LC cells degenerate, there would be a resulting increase in extraneuronal NM deposition which would also be associated with markers of inflammation in the LC.

4.4.1 Locus coeruleus cells

My findings show that the number of LC cells decreased with AD progression, with cell number being relatively preserved in the earlier Braak stages (0-II), but reducing dramatically after Braak stage III-IV, which is generally when cognitive symptoms appear usually defined as prodromal AD, see Figure 52. On average there was a 23% decrease in neuronal cell number between controls and those with prodromal AD and a further 65% decrease in neurons between those with prodromal AD and those with AD; this stepwise reduction in cell number is in line with previous research [113,132]. Interestingly the surviving LC cells were not typically observed to be bigger, more swollen or misshaped in the later AD stages as previously described [198].

There was a corresponding decrease in DβH expression, which again was only significant after Braak stage III and correlated highly with the number of LC cells. Although the DβH antibody detected noradrenergic activity in both axons and LC cells, this observation suggests that the decreased DβH expression is driven by the reduction in LC cell number. TH expression was also decreased in Braak group V-VI compared to controls but there was no significant reduction between group III-IV and V-VI and therefore DβH expression was more tightly correlated with the number of LC cells. Although previous studies have used both DβH and TH as markers of noradrenergic activity [110,116,131], my study suggests that DβH is the best marker for counting noradrenergic cells in the LC.

Age at death did not correlate with LC cell number even in the control group. This is not surprising as previous findings regarding an age effect on LC integrity have been mixed [111,114,123]. Generally, an age related decrease in LC cells has only been reported when comparing very old to young brains [108,112,116] whereas there was not such variation in age range in the cohorts used in my study.

4.4.2 Alzheimer's disease pathology

As previously reported both Aβ and p-tau increase in the temporal cortex with disease progression, as defined by the Braak stage, and in this study these two pathologies also correlated with each other. In the LC, Aβ is deposited at a lower level than in the temporal cortex. Although the trend is for Aβ to increase with Braak group in the LC, a significant difference could only be seen between Braak groups 0-II and V-VI with very little Aβ accumulation seen until Braak stage V. This suggests that Aβ deposition in the LC is not an early feature of AD and that it is not a key player in LC degeneration and is in agreement with previous research [209].

P-tau on the other hand is detected more readily in the LC than the temporal cortex at Braak stage 0-II, though many LC cells are still spared from p-tau accumulation at this stage. This is consistent with previous studies that have reported p-tau to accumulate in the LC from a young age and before NFTs are observed elsewhere in the cortex [133,215]. Tau pathology significantly increases in the LC with disease progression and is already observed at significantly greater amounts in incipient AD (Braak group III-IV) [44] compared to controls, as previously described[211,212]. Furthermore, tau pathology continues to increase despite the loss of LC cells suggesting that the surviving LC cells and axons contain high amounts of tau. From Braak stage III onwards however more p-tau can be detected in the temporal cortex than in the LC, both due to the loss of LC cells and the high number of neurons in the grey matter of the temporal cortex.

The literature suggests that p-tau accumulation in the LC begins decades before cell death [133] and in this study, p-tau accumulation in the LC was not associated with a reduction in cell number, suggesting p-tau is not the cause of LC cell loss. In the temporal cortex, both p-tau and Aβ accumulation are related to the LC cell loss, consistent with the hypothesis that changes in the LC impact on the cortical pathology at least in the temporal cortex as this region does not provide input to the LC.

Neuropil degeneration was only observed in the temporal cortex at Braak stages V-VI as expected. Indeed it is known that cortical atrophy occurs in AD, particularly in the temporal cortex, but this is not detected until symptoms are pronounced [324]. On the other hand, AD severity had no effect on neuropil degeneration in the LC. This was surprising as it was predicted that cell loss occurring in the LC would be associated with greater neuropil degeneration and that there would be spaces in the neuropil where LC cells were previously. However, the absence of neuropil degeneration is consistent with the absence of increased CD68 activity, a marker of microglial phagocytosis. Despite the absence of neuropil degeneration, the LC demonstrated cell alterations as discussed below.

4.4.3 Microglial changes

In the LC, there was a trend for Iba1 expression to increase with disease progression which reached significance only in the last stages of the disease. Iba1 is a homeostatic marker of microglia and its expression has been observed to be upregulated by activated microglia in experimental models of AD [325], but this has not been reported in the human cortex in AD [326]. Iba1 expression in the LC was associated with accumulation of p-tau accumulation and extraneuronal NM, but not with LC cell loss. Of note, Iba1 expression was higher in the more advanced stages of AD, while p-tau and extraneuronal NM increases were observed at the earliest stage (Braak stage III-IV). This implies that p-tau deposition and loss of NM by LC cells may precede the brain inflammatory response.

Surprisingly, there was little change in expression of HLA-DR or CD68 in the LC with disease progression. These markers of activated microglia are associated with the development of dementia [325]. Therefore, the changes of microglia in the LC appear to be more towards a physiological (higher expression of Iba1) rather than a pathological phenotype (CD68, HLA-DR). Microglial dysfunction has been implicated in AD [95] but looking at two different brain regions, both affected by the pathology, highlights the complexity and heterogeneity of the microglial response [327].

On the contrary, the temporal cortex presents no inflammatory change in relation to disease progression or protein accumulation in my study. This contradicts a human post-mortem study which found activated microglia to be associated with increased Aβ and tau pathology in AD in the cortex, but not in the subcortex [78]. However, the authors investigated subcortical structures other than the LC and activated microglia were assessed by visual appearance rather than by protein expression. If p-tau was driving Iba1 expression in the LC, we would expect to see the same pattern in the temporal cortex. Furthermore, whilst there were no differences in the extent of Iba1 expression between the two brain areas in the control cases, Iba1 expression was greater in the LC compared to the temporal cortex at both mild and severe AD stages. This suggests that it is the link between extraneuronal NM and inflammation that should be examined more closely.

4.4.4 Neuromelanin

Neuromelanin expression in the LC, decreased during the course of the disease and this decrease occurs very early in the disease process. The average amount of NM within each LC cell did not differ between controls and those in Braak group III-IV suggesting that the reduction in NM is driven by the loss of LC cells and not by a loss of pigment within the cells. Yet LC cell loss was not significant between controls and prodromal cases suggesting that NM loss is initially more significant than or somehow precedes cell loss. Therefore, the most heavily pigmented LC cells must degenerate first, whilst at the later stages, the surviving LC cells seem to contain less NM as reported in my study, see Figure 56.

Of note, whilst overall NM load diminished with increasing disease severity, the amount of extraneuronal NM increased with the Braak stage. Though observations of extraneuronal NM deposits in the LC have been reported in previous studies [196,199,200], this has never been quantified. It has been hypothesised that as LC cells degenerate, NM which is insoluble would be left in the extracellular space where it is likely to have a toxic effect on nearby neurons if it cannot be readily phagocytosed or degraded [143]. Certainly, in this study, extraneuronal NM was negatively associated with LC cell number. Previous research examining the effects of NM on cell cultures suggests that extraneuronal NM deposits will provoke an inflammatory reaction [261,262]. Here I observed Iba1 expression was associated with increased extraneuronal NM. This suggests microglia might have been recruited and/or activated in the attempt to remove extraneuronal NM, but this was not supported by a higher expression of phagocytic CD68+ microglia. The role of microglia in the LC remains to be explored further. Extraneuronal NM accumulation was also associated with Aβ and p-tau deposition, supporting a link between LC cells and the development of pathology.

This study had the benefit of employing an unbiased automated method with immunostaining calculated as a percentage of the total area, rather than involving manually counting of neurons. In conclusion as AD progresses, LC cell number decreases while Aβ, p-tau and extraneuronal NM deposition appears in the LC, which is accompanied by reactive microglia by the end stages of the disease. Our observations also highlight that p-tau and extraneuronal NM deposition are the earliest pathologies to occur in the LC. In the temporal cortex whilst Aβ and p-tau accumulation increase with disease progression, there were little changes in terms of inflammation.

To my knowledge, this was the first study to quantify extraneuronal NM deposition and to assess the deposition in relation to the features of AD pathologies (Aβ, p-tau and microglia). It is hypothesised that NM deposition accumulates as LC cells degenerate and this will lead to inflammation, exacerbating LC death. However, a causal relationship cannot be inferred from my findings and the relationship between NM accumulation and p-tau or Aβ accumulation both within the LC and in the temporal cortex requires further investigation.

4.4.5 Mesoscale discovery assay

In the LC, there was a decreased expression of four chemokines, MIP 1β, MDC, TARC and IL-8, in samples from Braak stages V-VI compared to tissue from control brains with little tau pathology, Braak stage 0-II. As these chemokines typically induce chemotaxis recruiting immune cells to inflammatory sites, these findings could indicate that microglia in the LC are not functioning as well in AD groups compared to controls. Yet this finding was not observed in the temporal cortex.

Previous literature shows inflammatory chemokines to be upregulated in AD and most studies focus on the association with amyloid, suggesting that Aβ acts as a stimulus for chemokine secretion. As observed in this study, there is little amyloid accumulation in the LC until Braak stage V-VI so if amyloid was influencing chemokine secretion we would expect to see an increase at this stage of disease rather than a decrease. Chemokine expression in the temporal cortex would also be expected to increase as amyloid accumulates.

Unfortunately, there was little clinical information to accompany the samples, such as whether those donating the samples had experienced any infections at or shortly prior to death as a current infection at the time of death may have influenced chemokine regulation. Additionally, the methodology was not as a robust as we would have liked. I was unable to solely isolate the LC from the tissue provided and so had to homogenise tissue that included not only the LC, but the surrounding pons. Therefore, the results included concentrations of markers from both the LC and pons which may have affected the findings. All samples underwent the same processing

however so it would be hoped that if there were differences that they would have still been detected.

4.4.6 Limitations

A limitation of this study was that the tissue had to be treated with hydrogen peroxide for 21 hours in order to bleach the brown neuromelanin pigment from the LC sections so that the pigment could be distinguished from the brown DAB chromogen which was used to visualise the various proteins of interest. Unfortunately, in some cases this damaged the thin sections and samples could not be used in the analysis.

NM is not present in rodent LC cells and in studies with human post-mortem tissue, many do not report bleaching of NM in their methods and do not discuss the presence of NM as an issue in their data analysis. However the process by which protein load is quantified is rarely fully reported. Where DAB staining and intracellular NM appear in the same cell, the staining is masked by the NM [328] and from the initial immunostaining completed as part of this project it is difficult to understand how DAB staining could be reliably analysed in sections containing NM[329,330]. As it was hypothesised that DAB staining of both Aβ and tau would increase, whereas LC cells containing NM would decrease with increased Braak stage, the similar appearance of NM to the DAB staining was likely to confound the results. There were a number of suggestions in the literature as to how to overcome this issue. A different chromogen could be used such as alkaline blue phosphatase [211] or AEC, however this chromogen has the disadvantage of non-specific staining. The amount of NM could be quantified for each case and then subtracted from the total staining, however as these markers would be assessed on separate slides this would only give a rough estimate of NM which is not very exact. Additionally, there is likely to be co-localisation of NM staining with some of the proteins of interest. Using hydrogen peroxide was determined to be the best method to remove NM without having to modify the IHC protocol and various tests were conducted in order to optimise the bleaching technique before conducting the study (Appendix F).

I did not find any age effect on the number of LC cells, however all cases examined as part of this study had an average age of death of approximately 85 years. It is known that p-tau accumulates at an early age, and therefore as I wanted to look at pathological changes in the initial progression of the disease it would have been useful to examine younger cases, but this was not part of the initial study.

The cases in this study were stratified by Braak stage as a marker of disease severity. However, there was no clinical information regarding cognition at time of death. It would have been useful

if cognitive assessments or more data surrounding duration of illness, overall atrophy, or APOE genotype had been available as additional measures of AD severity to compare the markers against. However clinical diagnosis was known and when cases were grouped by clinical diagnosis as opposed to histopathological diagnosis, the findings were still significant. By studying an older population many will have co-morbidities and mixed pathology in the brain. Whilst those with significant vascular pathology were excluded from the study, many will have some cerebral vascular changes, cerebral amyloid angiopathy or Lewy bodies as well as other diseases/inflammation. However these are real people and reflect the LOAD cases seen in society, so it may be that we need larger sample sizes rather than 'purer' samples.

Chapter 5 Conclusion

5.1 Overall discussion

This project has successfully demonstrated that LC degeneration and decline in overall NM load in AD evident at post-mortem, can be visualised using NM-MRI as a loss of signal in vivo. Although the MRI study had the advantage of allowing the entire LC volume to be examined, differences were only significant at the middle aspect of the LC which was subsequently the level also sampled in the human post-mortem study.

In the MRI study LC signal was significantly reduced between controls and mild AD participants, yet there was no difference in signal between those with mild AD and moderate AD. Whilst in the post-mortem study, LC cell loss was detected at the end stage of disease with no difference observed between the controls (Braak stage 0-II) and those with mild AD (Braak stage III-IV). These findings initially appear to contradict each other as differences in LC integrity are seemingly observed earlier on the MRI scans. However the neuromelanin findings in the post-mortem study, mirror the MRI study with an initial significant decrease between controls and those with prodromal/mild AD and then no difference in the amount of neuromelanin between those with mild AD and those with moderate to severe AD. It is proposed that the LC cells containing the highest concentrations of NM degenerate first in the early stages of AD. As the NM-MRI is detecting neuromelanin signal rather than LC cell loss this is reassuring. It suggests that the method of using the LC-CR despite not generating an absolute value for LC integrity is still an effective one.

It is difficult to evaluate how effective NM-MRI is as a biomarker for early detection of AD as participants with MCI were not included in this study. The post-mortem study saw a significant increase in Aβ accumulation in the temporal cortex between controls and those with mild AD whereas a significant reduction in LC cell number was only observed between mild AD and moderate AD groups. This suggests Aβ accumulation in the cortex occurs prior to LC cell loss and therefore amyloid biomarkers are relevant when it comes to early detection of AD. However, NM-MRI could potentially be an even earlier biomarker and less invasive than a PET scan, as early reduction in NM expression was observed both by MRI and post-mortem as discussed above. The only way to test this will be to perform both amyloid PET imaging and NM-MRI imaging within-subjects and follow them longitudinally to see which is a better predictor of cognitive decline. As neuropathology is known to occur decades prior to symptoms, biomarkers for early detection are essential to identify people for clinical trials and will be key to treatment.

The post-mortem study revealed a novel finding in that the amount of extracellular NM increases with disease progression, observed early in the disease process, and is most severe in the late stage. This might explain why there is no change in the overall NM load between groups III-IV and V-VI despite significant LC cell loss. Similarly, it may be that the NM-MRI is picking up signal from extracellular NM and this could be why the clinical study failed to find a difference in LC signal between the mild and moderate AD groups. There is currently no way of differentiating whether NM signal is intra or extraneuronal using MRI, but this seems a plausible explanation for my findings. Although the mechanism behind LC cell loss is still unclear, my findings are in support of the hypothesis that when LC cells degenerate (possibly firstly those that contain high concentrations of NM), the NM which is insoluble is unable to be removed and extraneuronal NM deposits start to accumulate. These deposits are likely to be toxic to surviving LC cells, therefore playing a role in further degeneration of these cells [260,261]. The accumulation of extracellular NM in the LC has not previously been quantified at post-mortem and may play a causal role in the selective vulnerability of LC neurons in AD.

Caution does need to be applied when trying to draw conclusions by looking at the combined data from these projects as of course it is not a within-subjects design and the groups examined in the MRI study i.e., controls, mild AD and moderate AD based on clinical diagnosis and cognitive assessment are not identical to the groups used in the human post-mortem project i.e., Braak 0-II, III-IV and V-VI based on Braak staging of tau pathology. Unfortunately, cognitive data was not available for the post-mortem samples to separate them in the same way, similarly it was impossible to know at what Braak stage the participants in the *in vivo* study had without doing a tau PET assessment. Braak group III-IV will include both those with prodromal/non-symptomatic AD as well as those with mild AD. Similarly group V-VI would include both those with moderate AD and severe AD. Whereas the MRI study did not knowingly include those with non-symptomatic AD or those with severe AD. Due to individual variation, the only way to directly compare MRI with post-mortem data would be to use a within-subjects design which is not possible. Even following subjects longitudinally is unlikely to be beneficial because people may live for years, even decades with their diagnosis and so post-mortem data would unlikely be reflective of an MRI scan unless performed close to death. Although it would have been useful to include an MCI or prodromal group in the MRI study, unfortunately funding was not granted to look at this group, and therefore stratifying the groups in this way was decided to be the most meaningful.

One of the areas that lacks understanding in AD is the interplay between Aβ and p-tau accumulations. Unfortunately, the MRI study provides no further information on this as neither tau nor amyloid biomarker status was known for the in vivo participants. The post-mortem study

is consistent with the literature showing early accumulation of tau in the LC which increases with disease progression and correlates with increases in p-tau in the cortex. Although initially the p-tau load is greater in the LC than the temporal cortex prior to cognitive symptoms, this study does not provide any further insight on how tau spreads from the LC to the cortex.

It has previously been supposed that because tau stabilises axonal microtubules, sustained production of abnormal tau will eventually affect the LC axons and cause them to degenerate eventually leading to cell death. It may take many years for this to occur, and it is known that tau first starts to accumulate in the LC in early adulthood. This study did not show a significant correlation between increasing p-tau in the LC and cell loss (though the trend was in the right direction), however cell loss would also mean a loss of tau and so conclusions about a causal relationship cannot be made. A recent animal study involving a tau insertion in the rat LC to mimic the pre-tangle stages of AD resulted in NET and DβH fibre density loss, the spread of tau along axons and impaired performance on an odour discrimination task in the absence of amyloid [331]. Aβ accumulation is not seen in the LC until late in the disease, so is it likely to participate in accelerating tau progression? Tau accumulation is known to be a better predictor of cognitive decline. However, most people have subcortical tau in the LC in mid-life, but not all go on to develop AD. In addition, Aβ accumulation in the cortex occurs earlier in the disease process and is currently the earliest detectable biomarker. This study finds no link between Aβ and LC cell loss within the LC. A recent study looking at LC integrity in people with genetic mutations for AD, shows that these people undergo LC cell loss and that *in vivo,* LC signal from the rostral aspect of the LC is attenuated which does suggest that somehow increased amyloid deposition affects LC integrity [332]. However, it is still unclear whether these things happen independently of each other rather than one causing the other.

This study also set out to examine the role of inflammation in LC degeneration in AD. Previous findings of increased Iba1 expression in the temporal cortex in AD have been inconsistent [333] and similarly were not observed in this study. However in contrast to current literature which often observes increases in activated phagocytic microglia in the temporal cortex in AD [333], no changes in CD68+ cells were observed between the Braak groups, despite Aβ and p-tau being increased. Similarly, no differences between Braak groups were seen in the multiplex assay performed in the temporal cortex. This challenges the idea that inflammation is the link between amyloid and tau. An increase in Iba1 load in the LC with increased Braak stage was observed and associated with both p-tau and extraneuronal NM accumulations, but as these Iba1 changes were only observed in the LC and not in the temporal cortex, the changes were attributed to the extraneuronal neuromelanin and LC degeneration. The phenotypes of microglia around NM deposits should be examined more closely. The phagocytic ability of microglia did not increase despite more

toxins/abnormal proteins in the brain, implying that microglia are either less efficient in clearing away NM, not activated by NM despite being reported to be toxic or that the immune system in people developing AD is impaired. This is consistent with evidence from GWAS in which genes associated with the immune system are risk factors for AD [95]. No changes in peripheral markers of inflammation were observed in the clinical study but unfortunately inflammatory changes cannot be examined on the MRI scan.

In summary, LC degeneration occurs in AD and NM-MRI could be a promising biomarker for early detection, but indirect measures of LC activity e.g., pupillometry are not currently sufficient to aid diagnosis. However, the role of amyloid, tau and inflammation in AD is still not well understood. How extraneuronal neuromelanin relates to LC cell death and the inflammatory reaction it may evoke requires further research as does the impact of reduced noradrenaline as a result of LC dysfunction. It may be that different types of intervention may be beneficial at different times in the disease i.e., at the start it may be more important to prevent Aβ forming, then to regulate microglia and once cognitive symptoms are evident it may be about reducing p-tau. Similarly there will likely be an optimum time to administer noradrenaline [28]. No work has yet been established as to how extraneuronal neuromelanin deposits could be removed.

5.2 Future work

Completing this PhD project has given me a general understanding of brain imaging and IHC, allowing me to develop my expertise from a psychology background (which largely involves assessing cognition and administering psychometric and cognitive assessments) and become more rounded as a scientist.

Data was obtained as part of the MRI clinical project that is still yet to be analysed but was beyond the scope of this PhD project. For example, resting state fMRI images were obtained to assess functional connectivity changes between the LC and other brain regions/networks in AD and a SWI sequence was performed to examine links between iron deposition and LC integrity. Additionally, LC signal could be compared to other general measures of brain atrophy such as total brain volume, white matter vs grey matter volume or hippocampal volume to assess any association.

In the post-mortem study, IHC could be performed for additional markers of inflammation and pro-inflammatory cytokines following the same protocol used in this project. Next steps would be to co-stain LC slides for microglial markers and extraneuronal NM to see what effect NM is having on inflammation in vivo in AD. To my knowledge there is currently no published literature on this,

only a few incidental observations made in studies. Do extraneuronal NM deposits attract microglia? Do they phagocytose it? Can we quantify this in AD?

A greater signal was unexpectedly observed from the RLC compared to LLC on NM-MRI even though previous post-mortem research has not shown differences in LC cell loss between the right and left sides. Unfortunately, this post-mortem project is unable to answer any questions about lateralisation differences as generally only one side of the LC was provided to sample. It would have been helpful to see if there are any differences in NM deposition either within or outside LC cells to see if this is causing the effect seen in MRI.

Finally, for both studies it would be useful to expand them to include younger cognitively unimpaired subjects to look at natural aging differences, as well as people with PD to see if my findings are unique to AD and not seen in other neurodegenerative diseases.

For the research area as a whole, to really know the validity of imaging the LC as a biomarker for AD, longitudinal NM-MRI studies are needed to test how effective LC signal is as a predictor of cognitive decline and how early in the disease process LC changes can be observed, by including subjects with mild cognitive impairment and those who are cognitively normal at entry with risk factors for AD. Funding applications are being made to pursue this based on the preliminary data from this pilot study.

Although current literature suggests that LC degeneration results in lower cortical levels of NA, it was unfortunate to not have a mechanism to measure this in the post-mortem study to examine how early NA loss occurs and to look for associations with the other markers examined. Similarly combining LC signal with noradrenergic PET tracers and other AD imaging biomarkers i.e. Aβ and Tau PET within subjects would be informative.

Both the MRI study and post-mortem studies continue to indicate significant LC degeneration in AD suggesting that pharmacological treatment to either increase the amount of NA or increase NA transmission may have the potential to restore the LC-NA pathway. So far only limited clinical trials in humans have been conducted with little success.

Trials involving MAO inhibitors, e.g. Sembragiline and Ladostigil have been unsuccessful in preventing people with MCI going on to develop AD [334]. However a recent proof of concept, phase 2 study of Rasagiline in people with AD found it to be associated with a slower decline in glucose metabolism as measured by FDG-PET and better performance on some of the cognitive tests compared to those on placebo [335].

A trial of Atomoxetine, a NA reuptake inhibitor, found no improvement in cognition measured by the ADAS-Cog compared to placebo in those with mild to moderate AD [336], but has not yet been explored in MCI populations. A Phase 3 clinical trial to test the effects of Guanfacine, an α2-AR agonist, plus AChEIs vs placebo in people with AD is currently underway at Imperial College London as well as a trial of Prazosin, an α1-AR antagonist to treat agitation in AD. The results of these studies or similar future trials are awaited with anticipation because these drugs are well tolerated [336] and it may be that if they are introduced at the right time that they may be able to help slow disease progression.

Appendix A Publications

1) Beardmore, R., Hou, R., Darekar, A., Holmes, C., and Boche, D. The Locus coeruleus in Aging and Alzheimer's Disease: A Post-mortem Brain Imaging Review *Journal of Alzheimer's Disease* 2021, 83(1): 5-22.
2) Hou, R., Beardmore, R., Holmes, C., Osmond, C and Darekar, A. A case-control study of the Locus Coeruleus degeneration in Alzheimer's Disease. *European Neuropsychopharmacology* 2021, 43: 153-159.

A research paper to present the findings from the post-mortem study is in preparation.

Appendix B Presentations

I have disseminated findings from this project either by oral or poster presentation at the following conferences and meetings:

Name of meeting/conference	Date	Presentation
Pint of Science, University of Southampton, public event	16/05/2017	Oral
MRI symposium, Medical Physics, UHS NHSFT	05/07/2017	Oral
Ageing and Dementia Event, University of Southampton	12/01/2018	Poster
Alzheimer's Research UK South Coast science café, public event	15/01/2018	Oral
MRI study day, Guerbet and UHS	31/01/2018	Oral
Psychopharmacology Meeting, University of Southampton	02/02/2018	Oral
Dementia Action Research & Education (DARE) network, University of Southampton	07/02/2018	Oral
Population health: Can research improve outcomes? Southern Health NHS FT	30/01/2019	Poster (Prize winner)
Alzheimer's Research UK, South coast/Sussex University	31/01/2019	Oral
Alzheimer's Research UK Annual Conference, Harrogate	26/03/2019	Poster
Southampton Medical and Health Research Conference, University of Southampton	11/06/2019	Poster
Early Career Researchers, Alzheimer's Research UK South Coast, Liphook	13/06/2019	Oral (Prize winner)
Psychopharmacology Meeting, University of Southampton	06/12/2019	Oral
IDEAC/NIHR Southampton	04/02/2020	Oral (Prize winner)
British Neuropathological Society conference	04/03/2020	Poster
Alzheimer's Association International Conference (AAIC) online	28/07/2020	Poster
Early Career Researchers, Alzheimer's Research UK South Coast, online	11/12/2020	Oral

Appendix C Study Materials used in clinical study

- Ethics approval letter from the Research Ethics Committee and Health Research Authority
- Control participant information sheet and consent form
- Participant information sheet and consent form
- Study partner consent form
- Case report forms used to record all data obtained as part of this study
- Validated psychometric and neuropsychiatric scales used:
    1. Standardised Mini Mental State Examination (SMMSE)
    2. Montreal Cognitive Assessment (MoCA)
    3. Alzheimer's Disease Assessment Scale-cognitive sub-scale (ADAS-COG)
    4. Pittsburgh Sleep Quality Index (PSQI)
    5. Alzheimer's Disease Cooperative Study Activities of Daily Living Scale (ADCS-ADL)
    6. Neuropsychiatric Inventory (NPI)

NHS
Health Research Authority

North West - Haydock Research Ethics Committee
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0207 104 8012

Please note: This is an acknowledgement letter from the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

03 October 2016

Dr Ruihua Hou
Senior Research Fellow
University of Southampton
Department of Psychiatry
Academic Centre, College Keep
4-12 Terminus Terrace, Southampton
SO14 3DT

Dear Dr Hou

Study title:	**Gaining new insight into the pathogenesis of Alzheimer's disease: investigating the role of the locus coeruleus using neuromelanin-sensitive MRI**
REC reference:	**16/NW/0675**
Protocol number:	**n/a**
IRAS project ID:	**209654**

Thank you for your submission of 27 September 2016. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 26 September 2016

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Other [Participant information sheet]	2	27 September 2016

Approved documents

The final list of approved documentation for the study is therefore as follows:

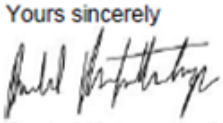
Document	*Version*	*Date*
Covering letter on headed paper [Covering letter]	1	02 September 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance letter]		01 September 2016
GP/consultant information sheets or letters [Patients GP letter]	1	21 July 2016
Interview schedules or topic guides for participants [Testing schedule]	1	21 July 2016
IRAS Application Form [IRAS_Form_08092016]		08 September 2016
Letter from funder [Funder letter]		21 February 2016
Letter from sponsor [Sponsor letter]		01 September 2016
Letter from statistician [Statistician letter]	1	21 July 2016
Other [Control PIS]	1	21 July 2016
Other [Control consent form]	1	21 July 2016
Other [Study partner consent form]	1	21 July 2016
Other [Control GP letter]	1	21 July 2016
Other [Abnormal findings GP letter]	1	21 July 2016
Other [Participants debrief letter]	1	21 July 2016
Other [Questinnairs1 ADAS cog]		30 June 2014
Other [Questionnaire 2 ADL]		23 July 2014
Other [Questionnair 3 NPI]		
Other [Questionnair 4 PSQI]		
Other [Questionnair 5 SMMSE]		
Other [Questionnaire Demographic]	1	21 July 2016
Other [Questionnaire demographic control]	1	21 July 2016
Other [Questionnair medication]	1	21 July 2016
Other [Questionnaire medical history]	1	21 July 2016
Other [Questionnaire MRI checklist]	1	21 July 2016
Other [Participant information sheet]	2	27 September 2016
Participant consent form [Patients Consent Form]	1	21 July 2016
Referee's report or other scientific critique report [Referee report]		12 July 2016
Research protocol or project proposal [Research Protocol]	1	21 July 2016
Summary CV for Chief Investigator (CI) [CI CV]		07 July 2016
Summary CV for student [Student CV]		13 July 2016
Summary CV for supervisor (student research) [supervisor CV]		11 January 2016
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Study flow-chart]	1	21 July 2016

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all

participating sites.

16/NW/0675	Please quote this number on all correspondence

Yours sincerely



Rachel Katzenellenbogen
REC Manager

E-mail: nrescommittee.northwest-haydock@nhs.net

Copy to: Ms Diana Galpin, University of Southampton
Ms Penny Bartlett, Southern Health NHS Foundation Trust

UNIVERSITY OF Southampton
School of Medicine

Southern Health NHS
NHS Foundation Trust

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Tel: 02380 475206
Fax: 02380 463022
www.southernhealthft.nhs.uk

Control Participant Information Sheet

Full Study Title:	Gaining new insight into the pathogenesis of Alzheimer's disease: investigating the role of the locus coeruleus using neuromelanin-sensitive MRI
Brief Title:	Investigating the Locus Coeruleus in Alzheimer's Disease using MRI
Sponsor:	University of Southampton

You are being invited to take part in a research study. Before deciding to participate it is important for you to understand why the research is being done and what it will involve. This information sheet has been written to tell you why this study is being done and what will be required of you if you decide to take part. It will also tell you what the potential benefits and risks of taking part are to enable you to make an informed decision about whether you would like to take part in this study.

Please take your time and read this information sheet carefully. You may discuss this study with your family, friends, GP or others involved in your care if you find it helpful in making your decision. If anything you read is not clear or you would like more information, please do not hesitate to contact a member of the research team who will be able to answer any questions you may have.

Thank you for taking the time to read this information sheet and for considering taking part.

Who is conducting the study?

This study is conducted by researchers from the Faculty of Medicine, University of Southampton. Some of the data from this study will be collected and used as part of an educational qualification. Our research team include;

Dr Ruihua Hou, Senior Research Fellow in Psychiatry
Dr Angela Darekar, the lead for MRI Physics
Professor Simon Liversedge, Professor of Experimental Psychology
Professor Clive Holmes, Professor of Biological Psychiatry
Miss Rebecca Ollington, PhD student

What is the purpose of this study?

The locus coeruleus (LC) is a tiny nucleus in the brain stem. Evidence from animal and post-mortem studies suggest that the LC is damaged in Alzheimer's disease with some arguing that it is one of the first brain areas to be damaged. Recently, a new high-resolution Magnetic Resonance Imaging (MRI) technique has been developed which allows us to see this small structure on a brain scan. This pilot study aims to test how successful this new MRI technique is at detecting signal intensity in the LC in people with Alzheimer's disease and to examine the quality of these images. It aims to see whether there are differences in the LC signal on the MRI scans between people with Alzheimer's disease and age-matched healthy control subjects.

Changes in pupil size and responses to light and other physiological measures e.g. heart rate and blood pressure, have previously been used as indirect measures of LC activity. This study will therefore also look at the link between changes in the signal intensity of the LC as shown on the MRI scan and these other biological and physiological measures.

The LC produces an important chemical called noradrenaline (NA). Current treatments for Alzheimer's disease do not affect this chemical. This study will advance our understanding of the role of the LC and NA in Alzheimer's disease which may lead to the development of new treatment targets.

Why have I been invited?

You have been invited to take part in this study because you have no memory impairment and can act as an age-matched control subject in this study and have registered your interest in volunteering for research. Data from the control subjects will be compared to data from the Alzheimer's disease group.

Do I have to take part?

Participating in this study is entirely voluntary. You do not have to participate in the study and if you decide not to take part the standard of your medical care and your legal rights will not be affected.

If you do decide to take part we will ask you to sign a consent form to confirm that you understand the study and agree to take part. You will be given a copy of this consent form for your records.

After signing the consent forms you are still free to change your mind and you may withdraw from the study at any time and you do not have to give a reason for doing so.

What will happen to me if I take part? What will I have to do?

There will be a telephone screening stage and a total of 3 visits for testing sessions that you will need to attend.

During the screening stage, your eligibility and diagnosis will be reviewed and validated.

Visit 1 will last approximately 2 hours and will take place at the Memory Assessment & Research Centre, Moorgreen Hospital.

Study procedures at this visit include:

- discussing the study and signing the consent forms
- taking a medical history and reviewing your current medications;
- taking some physiological measures e.g. heart rate, blood pressure, respiration rate. These measures will be taken at rest, on standing, after doing mental arithmetic and after your hand has been in ice water for 90 seconds.
- performing an electrocardiogram (ECG) to measure the activity of your heart.
- giving a blood sample (approximately 15ml, equivalent to one tablespoon).
- performing some tests which assess your memory, thinking ability and sleep quality

Visit 2 will last approximately 1 - 1.5 hours and will take place at the imaging centre at Southampton General Hospital. You will undergo a MRI scan which involves lying still in a machine for up to 1 hour.

Visit 3 will last approximately 2 hours and will take place in the Psychology Department at the University of Southampton. You will be asked to complete a short reading test while your eye movement is recorded by a remote eye-tracker, in addition, your pupil size and responses will be recorded.

What will happen to the blood sample that I give?

A blood sample will be taken at the Memory Assessment & Research Centre. A total of 15ml (approximately 1 tablespoon) of blood will be taken. Usually all of the blood can be obtained at the same time which means you should not get an extra needle prick.

We would like to use the blood sample to investigate the blood cells that work as part of your immune system.

One of the blood samples will also be used for genetic testing. Information gathered from genetic testing may help researchers to investigate how it links to the LC signal intensity. At present, the results of genetic testing are difficult to interpret in terms of prognosis for an individual, and therefore you will not be told the results of the genetic testing. This is not an optional blood test, i.e. you will be required to have this blood test if you wish to take part in the study, and you will be asked to give consent for this to take place.

Blood samples will be analysed 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. Your samples will be destroyed according to the standard procedures of the laboratory as soon as possible after the study is completed and results are available to the Sponsor.

One of the samples taken will be kept for use in future research projects. This is an optional blood sample and you do not have to agree to do this in order to take part in this study. If you do give permission for this sample to be taken, the sample will be labelled with 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.

What are the possible disadvantages and risks of taking part?

Time and effort to attend testing sessions are the main disadvantages of taking part in this study. You will have rest periods during testing, if needed, and are free to stop any test or procedure at any time. Study staff will be fully trained to administer the neuropsychological tests and have testing experience within a clinical trials setting.

Taking blood samples:
During the study, blood will be drawn to perform a variety of tests. The risks of drawing blood include temporary discomfort from the needle in the arm, bruising, swelling at the needle site, and, in rare instances, infection.

Conduct of cognitive tasks:
The conduct of cognitive tasks could be tiring for some participants. You may experience nervousness, tiredness or boredom during the mental testing. Sufficient breaks will be provided between the tasks to minimise the effects of this. It is unlikely that you will experience emotional discomfort or distress as a result of conducting these tasks.

Undergoing MRI scan:
The MRI scan will last up to 45 minutes. Although the procedure is not associated with any pain, it can be loud which people sometimes find discomforting. You may feel slightly claustrophobic. Both the research team at the Memory Assessment and Research Centre and the imaging staff at Southampton General Hospital will go through a checklist to ensure a health and safety procedure is followed and there are no risks involved.

What are the possible benefits of taking part?

There are no other direct expected benefits to you taking part in this study but you may feel satisfaction in knowing that your participation may help others in the future. You will be contributing to a knowledge base of the role of the locus coeruleus in the development of Alzheimer's disease and your involvement may help to develop new intervention targets to improve clinical outcomes and quality of life of patients with Alzheimer's disease in future.

Your MRI brain scans will be reviewed by a radiologist. If any pathological findings are made i.e. if anything unusual is found, plans will be in place for you where further support or a referral is needed.

Who has reviewed the study?

The study has been reviewed by an independent Research Ethics Committee (REC Ref: 16/NW/0675). The members of the NHS Research Ethics Committee will assess all of the details of the study and every effort will be made during and after the study to ensure your safety and to ensure that the research team will respect and protect your rights, wellbeing and dignity throughout the study and after completion of the study. The NHS Research and Development team at Southern Health NHS Foundation Trust have also reviewed and approved this study.

What if there is a problem?

Southern Health NHS Foundation Trust as well as 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 of taking part in the study, you should contact the research team at the Memory Assessment and Research Centre in Southampton on **02380 475206** during office hours, or the on-call member of the Southampton research team on **07773 355969** if it is out of hours.

In the unlikely 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 treatment.

If you have any concerns about your participation in the study or wish to make a complaint, please contact Isla Morris, Research Integrity and Governance Manager, , Research Governance Office, Corporate Services, Building 37, Highfield Campus, Southampton SO17 1BJ. Telephone: 02380 595058 email: rgoinfo@soton.ac.uk .

Further information on your rights as a research subject and on the complaints procedure can also be provided by the Complaints and Patient Advice and Liaison Services (PALS) team, 5 Sterne Road, Tatchbury Mount, Calmore, Southampton, SO40 2RZ. Telephone: 02380 874065.

Will my taking part in the study be kept confidential?

You will be given a unique study number. All information recorded that is to be included in the analysis will be anonymised and coded using this number and your initials only. Your main clinical medical records will be stored at the Memory Assessment and Research Centre (Southern Health NHS Foundation Trust). These records will be kept in a locked cabinet in a locked office within a locked unit and will be kept confidential in line with the trust's information governance and data protection policies. All blood samples stored will be identifiable only by the unique study number.

MRI scans will be acquired as part of this research trial, but not as part of the diagnostic pathway. However, the standard structural MRI scans will be reviewed and reported by a Consultant Neuroradiologist, to check for any incidental findings. If any clinically significant findings are found, a procedure will be followed to manage this. This will involve a letter and report being sent to your GP, with the expectation that this will be followed up via the usual clinical management pathway. MRI data will be anonymised before being extracted from clinical systems for research analysis. This data will be stored securely for the duration of the study and archived in line with trust policy.

If you take part it will be necessary for qualified members of the University of Southampton, Southern Health NHS Foundation Trust, the Research Ethics Committee 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.

If the results are published all of your records will be kept confidential and your name will not be disclosed to anyone outside of the research team at MARC and the imaging site at the Southampton General Hospital.

Paper and other manual files will be archived securely and destroyed after a period of 15 years. Appropriate access controls will be in place to ensure that access to confidential research information is restricted to those who need access within the research team.

We will notify your GP about your participation in the study if you consent to this.

Early withdrawal from the study

The entire study could be discontinued at any time by the following entities: study doctors, the Ethics Committee, the University of Southampton or 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 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.

In the event of a loss of capacity, the research team would retain tissue and personal data collected and continue to use it confidentially in connection with the purposes for which consent is being sought. If you withdraw from the study the information you have already provided will be kept confidential.

What will happen to the results of the research study?

A written report detailing the results will be submitted to the regulatory authorities with the aim to publish them in a scientific journal. These publications and reports will not identify you. If you would like a copy of the results you can tell the researcher who will make them available to you at the end of the study after publication.

Expenses and payments

There will be no additional cost to you as a result of being in the study. There is no payment for

taking part in this study. Any travel expenses will be reimbursed.

Who is organising or sponsoring the research study?

The research is being organised by researchers from the Faculty of Medicine, University of Southampton and conducted at the Memory Assessment and Research Centre, Tom Rudd Unit, Moorgreen Hospital.

The University of Southampton is sponsoring this study and is responsible for the proper conduct of this study.

The study is being funded by Alzheimer's Research UK.

Contact details for further information

If you would like to discuss your potential involvement in this research further please contact:

Name: **Rebecca Ollington**
Email address: rebecca.ollington@nhs.net
Telephone: 02380 475206
Address: The Memory Assessment and Research Centre (MARC),
Tom Rudd Unit, Moorgreen Hospital, Botley Road, Southampton, SO30 3JB

University of Southampton
School of Medicine

Southern Health NHS
NHS Foundation Trust
Memory Assessment and Research Centre
Moorgreen, Tom Rudd Unit
Botley Road
West End
Southampton
SO30 3JB

Tel: 02380 475208
Fax: 02380 463022
www.southernhealthft.nhs.uk

Control Participant Consent Form

Full Study Title:	Gaining new insight into the pathogenesis of Alzheimer's Disease: investigating the role of the locus coeruleus using neuromelanin-sensitive MRI
Brief title:	Investigating the Locus Coeruleus in Alzheimer's Disease using MRI
Principal Investigator:	Dr Ruihua Hou, Faculty of Medicine, University of Southampton.

Protocol Number:	
Patient Identification Number for this trial:	

Please read the following statements. If any questions arise, please ask the researcher for more information. If you agree with a statement, please put your initials in the box on the right.

1	I have read (or have had it read to me) and understood the participant information sheet for the above study dated …… Version …….. I have had enough time to decide whether I would like to take part in the study.	
2	I understand that my participation is voluntary and that if I decide not to take part in this study that my medical care will not be affected. I understand that I am free to withdraw from the study at any time and that I do not have to give a reason for doing so and without my medical care or legal rights being affected.	
3	I have discussed the study with the research team and all of my questions have been answered in a way that makes sense to me and I have no outstanding questions.	

4	I understand why this research is being carried out and any foreseeable risks involved.	
5	I understand that where it is relevant, sections of my medical records and the data collected during the study may be looked at by individuals from the sponsor or their agents, by individuals from government or other regulatory authorities, or by individuals from the Southern Health NHS Foundation Trust. I give permission for these individuals to have access to my records.	
6	I 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. I agree that data gathered about me can be stored by the University of Southampton for possible use in future research projects.	
7	I agree to allow research staff to collect a blood sample (approximately 15ml, one tablespoon) from me. I understand how the sample will be collected, used and stored.	
8	I agree to allow researchers to do a genotyping test on the blood sample I give for this study. I understand I will not be given the results of this test.	
9	I agree to my sample being stored for future unspecified research studies with appropriate ethical approval and I have been made aware that this is **optional** to agree to.	
10	I agree to provide information about my memory, my thinking ability, my behaviour, and my everyday functioning.	
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 attend the Southampton General Hospital for a MRI brain scan.	
14	I agree to perform an eye tracking test.	
15	I agree to my GP being informed of my participation in the study.	
16	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.	
17	I understand that in the event of a loss of capacity, the research team would retain tissue and personal data collected and continue to use it confidentially in connection with the purposes for which consent is being sought.	
18	I understand that I will not benefit financially from this research or any future research using my data or samples.	

19	I agree to take part in the above study.	

Participant Name (PRINT):	Signature:	Date:

INVESTIGATOR

- *I have carefully explained to the participant 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.*

Person taking consent (PRINT):	Signature:	Date:

UNIVERSITY OF Southampton
School of Medicine

Southern Health NHS
NHS Foundation Trust

Memory Assessment and Research Centre
Moorgreen, Tom Rudd Unit
Botley Road
West End
Southampton
SO30 3JB

Tel: 02380 475206
Fax: 02380 463022
www.southernhealthft.nhs.uk

Participant and Study Partner Information Sheet

Full Study Title:	Gaining new insight into the pathogenesis of Alzheimer's disease: investigating the role of the locus coeruleus using neuromelanin-sensitive MRI
Brief Title:	Investigating the Locus Coeruleus in Alzheimer's Disease using MRI
Sponsor:	University of Southampton

You are being invited to take part in a research study. Before deciding to participate it is important for you to understand why the research is being done and what it will involve. This information sheet has been written to tell you why this study is being done and what will be required of you if you decide to take part. It will also tell you what the potential benefits and risks of taking part are to enable you to make an informed decision about whether you would like to take part in this study.

Please take your time and read this information sheet carefully. You may discuss this study with your family, friends, GP or others involved in your care if you find it helpful in making your decision. If anything you read is not clear or you would like more information, please do not hesitate to contact a member of the research team who will be able to answer any questions you may have.

Thank you for taking the time to read this information sheet and for considering taking part.

Who is conducting the study?

This study is conducted by researchers from the Faculty of Medicine, University of Southampton. Some of the data from this study will be collected and used as part of an educational qualification. Our research team include;

Dr Ruihua Hou, Senior Research Fellow in Psychiatry
Dr Angela Darekar, the lead for MRI Physics
Professor Simon Liversedge, Professor of Experimental Psychology
Professor Clive Holmes, Professor of Biological Psychiatry
Miss Rebecca Ollington, PhD student

What is the purpose of this study?

The locus coeruleus (LC) is a tiny nucleus in the brain stem. Evidence from animal and post-mortem studies suggest that the LC is damaged in Alzheimer's disease with some arguing that it is one of the first brain areas to be damaged. Recently, a new high-resolution Magnetic Resonance Imaging (MRI) technique has been developed which allows us to see this small structure on a brain scan. This pilot study aims to test how successful this new MRI technique is at detecting signal intensity in the LC in people with Alzheimer's disease and to examine the quality of these images. It aims to see whether there are differences in the LC signal on the MRI scans between people with Alzheimer's disease and age-matched healthy control subjects.

Changes in pupil size and responses to light and other physiological measures e.g. heart rate and blood pressure, have previously been used as indirect measures of LC activity. This study will therefore also look at the link between changes in the signal intensity of the LC as shown on the MRI scan and these other biological and physiological measures.

The LC produces an important chemical called noradrenaline (NA). Current treatments for Alzheimer's disease do not affect this chemical. This study will advance our understanding of the role of the LC and NA in Alzheimer's disease which may lead to the development of new treatment targets.

Why have I been invited?

You have been invited to take part in this study because you have a diagnosis of mild cognitive impairment or mild to moderate Alzheimer's disease and have registered your interest in volunteering for research.

Do I have to take part?

Participating in this study is entirely voluntary. You do not have to participate in the study and if you decide not to take part the standard of your medical care and your legal rights will not be affected.
If you do decide to take part we will ask you to sign a consent form to confirm that you understand the study and agree to take part. You will be given a copy of this consent form for your records.

After signing the consent forms you are still free to change your mind and you may withdraw from the study at any time and you do not have to give a reason for doing so.

What will happen to me if I take part? What will I have to do?

There will be a telephone screening stage and a total of 3 visits for testing sessions that you and your study partner will need to attend.

During the screening stage, your eligibility and diagnosis will be reviewed and validated.

Visit 1 will last approximately 2-2.5 hours and will take place at the Memory Assessment & Research Centre, Moorgreen Hospital.

Study procedures at this visit include:

- discussing the study and signing the consent forms
- taking a medical history and reviewing your current medications;
- taking some physiological measures e.g. heart rate, blood pressure, respiration rate. These measures will be taken at rest, on standing, after doing mental arithmetic and after your hand has been in ice water for 90 seconds. performing an electrocardiogram (ECG) to measure the activity of your heart.
- giving a blood sample (approximately 15ml, equivalent to one tablespoon).
- performing some tests which assess your memory, thinking ability and sleep quality

Visit 2 will last approximately 1-1.5 hours and will take place at the imaging centre at Southampton General Hospital. You will undergo a MRI scan which involves lying still in a machine for up to 1 hour.

Visit 3 will last approximately 2 hours and will take place in the Psychology Department at the University of Southampton. You will be asked to complete a short reading test while your eye movement is recorded by a remote eye-tracker, in addition, your pupil size and responses will be recorded.

What is your study partner required to do during the study?

You need a study partner to participate in this study. Your study partner does not need to be related to you, but should be someone who knows you well enough and spends enough time with you to be able to answer questions about your mood, thinking ability and everyday functioning. They will also need to accompany you to all three visits.

At Visit 1 which will last approximately 2-2.5 hours and will take place at the Memory Assessment and Research centre they will:

- discuss the study with research staff and sign the consent form
- aid in providing information about your medical history and current medications
- provide information about your mood, thinking ability, sleep quality and everyday functioning

What will happen to the blood sample that I give?

A blood sample will be taken at the Memory Assessment & Research Centre. A total of 15ml (approximately 1 tablespoon) of blood will be taken. Usually all of the blood can be obtained at the same time which means you should not get an extra needle prick.

We would like to use the blood sample to investigate the blood cells that work as part of your immune system.

One of the blood samples will also be used for genetic testing. Information gathered from genetic testing may help researchers to investigate how it links to the LC signal intensity. At present, the results of genetic testing are difficult to interpret in terms of prognosis for an individual, and therefore you will not be told the results of the genetic testing. This is not an optional blood test, i.e. you will be required to have this blood test if you wish to take part in the study, and you will be asked to give consent for this to take place.

Blood samples will be analysed 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. Your samples will be destroyed according to the standard procedures of the laboratory as soon as possible after the study is completed and results are available to the Sponsor.

One of the samples taken will be kept for use in future research projects. This is an optional blood sample and you do not have to agree to do this in order to take part in this study. If you do give permission for this sample to be taken, the sample will be labelled with 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.

What are the possible disadvantages and risks of taking part?

Time and effort to attend testing sessions are the main disadvantages of taking part in this study. You will have rest periods during testing, if needed, and are free to stop any test or procedure at any time. Study staff will be fully trained to administer the neuropsychological tests and have testing experience within a clinical trials setting.

Taking blood samples:
During the study, blood will be drawn to perform a variety of tests. The risks of drawing blood include temporary discomfort from the needle in the arm, bruising, swelling at the needle site, and, in rare instances, infection.

Conduct of cognitive tasks:
The conduct of cognitive tasks could be tiring for you. You may experience nervousness, tiredness or boredom during the mental testing. Sufficient breaks will be provided between the tasks to minimise the effects of this.

Undergoing MRI scan:
The MRI scan will last up to 45 minutes. Although the procedure is not associated with any pain, it can be loud which people sometimes find discomforting. You may feel slightly claustrophobic. Both the research team at the Memory Assessment and Research Centre and the imaging staff at Southampton General Hospital will go through a checklist to ensure a health and safety procedure is followed and there are no risks involved.

What are the possible benefits of taking part?

There are no other direct expected benefits to you taking part in this study but you may feel satisfaction in knowing that your participation may help others in the future. You will be contributing to a knowledge base of the role of the locus coeruleus in the development of Alzheimer's disease and your involvement may help to develop new intervention targets to improve clinical outcomes and quality of life of patients with Alzheimer's disease in future.

Your MRI brain scan will be reviewed by a radiologist. If any pathological findings are made, i.e. if anything unusual is found, plans will be in place for you where further support or a referral is needed.

Who has reviewed the study?

The study has been reviewed by an independent Research Ethics Committee (REC Ref:

16/NW/0675). The members of the NHS Research Ethics Committee will assess all of the details of the study and every effort will be made during and after the study to ensure your safety and to ensure that the research team will respect and protect your rights, wellbeing and dignity throughout the study and after completion of the study. The NHS Research and Development team at Southern Health NHS Foundation Trust have also reviewed and approved this study.

What if there is a problem?

Southern Health NHS Foundation Trust as well as 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 of taking part in the study, you should contact the research team at the Memory Assessment and Research Centre in Southampton on **02380 475206** during office hours, or the on-call member of the Southampton research team on **07773 355969** if it is out of hours.

In the unlikely 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 treatment.

If you have any concerns about your participation in the study or wish to make a complaint, please contact Isla Morris, Research Integrity and Governance Manager, Research Governance Office, Corporate Services, Building 37, Highfield Campus, Southampton SO17 1BJ. Telephone: 02380 595058 email: rgoinfo@soton.ac.uk .

Further information on your rights as a research subject and on the complaints procedure can also be provided by the Complaints and Patient Advice and Liaison Services (PALS) team, 5 Sterne Road, Tatchbury Mount, Calmore, Southampton, SO40 2RZ. Telephone: 02380 874065.

Will my taking part in the study be kept confidential?

You will be given a unique study number. All information recorded that is to be included in the analysis will be anonymised and coded using this number and your initials only. Your main clinical medical records will be stored at the Memory Assessment and Research Centre (Southern Health NHS Foundation Trust). These records will be kept in a locked cabinet in a locked office within a locked unit and will be kept confidential in line with the trust's information governance and data protection policies. All blood samples stored will be identifiable only by the unique study number.

MRI scans will be acquired as part of this research trial, but not as part of the diagnostic pathway. However, the standard structural MRI scans will be reviewed and reported by a Consultant Neuroradiologist, to check for any incidental findings. If any clinically significant

findings are found, a procedure will be followed to manage this. This will involve a letter and report being sent to your GP, with the expectation that this will be followed up via the usual clinical management pathway. MRI data will be anonymised before being extracted from clinical systems for research analysis. This data will be stored securely for the duration of the study and archived in line with trust policy.

If you take part it will be necessary for qualified members of the University of Southampton, Southern Health NHS Foundation Trust, the Research Ethics Committee 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..

If the results are published all of your records will be kept confidential and your name will not be disclosed to anyone outside of the research team at MARC and the imaging site at the Southampton General Hospital.

Paper and other manual files will be archived securely and destroyed after a period of 15 years. Appropriate access controls will be in place to ensure that access to confidential research information is restricted to those who need access within the research team.

We will notify your GP about your participation in the study if you consent to this.

Early withdrawal from the study

The entire study could be discontinued at any time by the following entities: study doctors, the Ethics Committee, the University of Southampton or 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 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.

In the event of a loss of capacity, the research team would retain tissue and personal data collected and continue to use it confidentially in connection with the purposes for which consent is being sought. If you withdraw from the study the information you have already provided will be kept confidential.

What will happen to the results of the research study?

A written report detailing the results will be submitted to the regulatory authorities with the aim to publish them in a scientific journal. These publications and reports will not identify you. If you would like a copy of the results you can tell the researcher who will make them available to you at the end of the study after publication.

Expenses and payments

There will be no additional cost to you as a result of being in the study. There is no payment for taking part in this study. Any travel expenses will be reimbursed.

Who is organising or sponsoring the research study?

The research is being organised by researchers from the Faculty of Medicine, University of Southampton and conducted at the Memory Assessment and Research Centre (MARC), Tom Rudd Unit, Moorgreen Hospital.

The University of Southampton is sponsoring this study and is responsible for the proper conduct of this study.

The study is being funded by Alzheimer's Research UK.

Contact details for further information

If you would like to discuss your potential involvement in this research further please contact:

Name: **Rebecca Ollington**
Email address: rebecca.ollington@nhs.net
Telephone: 02380 475206
Address: The Memory Assessment and Research Centre (MARC),
Tom Rudd Unit, Moorgreen Hospital, Botley Road, Southampton, SO30 3JB

UNIVERSITY OF Southampton
School of Medicine

Southern Health NHS
NHS Foundation Trust

Memory Assessment and Research Centre
Moorgreen, Tom Rudd Unit
Botley Road
West End
Southampton
SO30 3JB

Tel: 02380 475206
Fax: 02380 463022
www.southernhealthft.nhs.uk

Participant Consent Form

Full Study Title:	Gaining new insight into the pathogenesis of Alzheimer's Disease: investigating the role of the locus coeruleus using neuromelanin-sensitive MRI
Brief title:	Investigating the Locus Coeruleus in Alzheimer's Disease using MRI
Principal Investigator:	Dr Ruihua Hou, Faculty of Medicine, University of Southampton.

Protocol Number:	
Patient Identification Number for this trial:	

Please read the following statements. If any questions arise, please ask the researcher for more information. If you agree with a statement, please put your initials in the box on the right.

1	I have read (or have had it read to me) and understood the participant information sheet for the above study dated …… Version ……. I have had enough time to decide whether I would like to take part in the study.	
2	I understand that my participation is voluntary and that if I decide not to take part in this study that my medical care will not be affected. I understand that I am free to withdraw from the study at any time and that I do not have to give a reason for doing so and without my medical care or legal rights being affected.	
3	I have discussed the study with the research team and all of my questions have been answered in a way that makes sense to me and I have no outstanding questions.	

4	I understand why this research is being carried out and any foreseeable risks involved.	
5	I understand that where it is relevant, sections of my medical records and the data collected during the study may be looked at by individuals from the sponsor or their agents, by individuals from government or other regulatory authorities, or by individuals from the Southern Health NHS Foundation Trust. I give permission for these individuals to have access to my records.	
6	I 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. I agree that data gathered about me can be stored by the University of Southampton for possible use in future research projects.	
7	I agree to allow research staff to collect a blood sample (approximately 15ml, one tablespoon) from me. I understand how the sample will be collected, used and stored.	
8	I agree to allow researchers to do a genotyping test on the blood sample I give for this study. I understand I will not be given the results of this test.	
9	I agree to my sample being stored for future unspecified research studies with appropriate ethical approval and I have been made aware that this is **optional** to agree to.	
10	I agree for my study partner to provide information about my memory, my thinking ability, my behaviour, and my everyday functioning.	
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 attend the Southampton General Hospital for a MRI brain scan.	
14	I agree to perform an eye tracking test.	
15	I agree to my GP being informed of my participation in the study.	
16	I agree to my GP being informed of my memory test score and for my score to be included in my medical records. This information will be kept confidentially.	
17	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.	
18	I understand that in the event of a loss of capacity, the research team would retain tissue and personal data collected and continue to use it confidentially in connection with the purposes for which consent is being sought.	

19	I understand that I will not benefit financially from this research or any future research using my data or samples.	
20	I agree to take part in the above study.	

Participant Name (PRINT):	**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.*

Person taking consent (PRINT):	**Signature:**	**Date:**

UNIVERSITY OF Southampton
School of Medicine

Southern Health NHS
NHS Foundation Trust

Memory Assessment and Research Centre
Moorgreen, Tom Rudd Unit
Botley Road
West End
Southampton
SO30 3JB

Tel: 02380 475208
Fax: 02380 463022
www.southernhealthft.nhs.uk

Study Partner Consent Form

Full Study Title:	Gaining new insight into the pathogenesis of Alzheimer's Disease: investigating the role of the locus coeruleus using neuromelanin-sensitive MRI
Brief title:	Investigating the Locus Coeruleus in Alzheimer's Disease using MRI
Principal Investigator:	Dr Ruihua Hou, Faculty of Medicine, University of Southampton.

Protocol Number:	
Patient Identification Number for this trial:	

Please read the following statements. If any questions arise, please ask the researcher for more information. If you agree with a statement, please put your initials in the box on the right.

1	I have read (or have had it read to me) and understood the participant information sheet for the above study dated……, Version …….. I have had enough time to decide whether I would like to take part in the study.	
2	I understand that my participation is voluntary. I understand that I am free to withdraw from the study at any time and that I do not have to give a reason for doing so and without my medical care or legal rights being affected.	
3	I have discussed the study with the research team and all of my questions have been answered in a way that makes sense to me and I have no outstanding questions.	

4	I understand why this research is being carried out and any foreseeable risks involved.	
5	I confirm that I am in regular contact with the patient.	
6	I agree to provide information about the patient's memory, behaviour and abilities at home	
7	I understand that if I cannot fulfil the study responsibilities I should let the research team know. I will try to accompany the patient to all visits. If for any reason I cannot attend the study visits with the patient I will try to find someone else who can take over this responsibility.	
8	If the patient becomes unwilling to attend any of the study visits I will inform study staff.	
9	I understand that if the patient loses the ability to fully consent to their involvement in this study that both the patient and my participation will be stopped immediately with no further study procedures carried out.	
10	I understand that I will not benefit financially from this research or any future research using the information I have provided.	
11	I agree to take part in the above study.	

Participant Name (PRINT):	**Signature:**	**Date:**

INVESTIGATOR

- *I have carefully explained to the study partner the nature and purpose of the above study.*
- *There has been an opportunity for the study partner to ask questions about this research study.*
- *I have answered all questions that the study partner has about this study.*

Person taking consent (PRINT):	**Signature:**	**Date:**

IMLOC	
Subject No.	
Pt. initials	
Date	
Assessor initials	

IMLOC: Demographics CRF

Study Partner Information

Relationship of study partner to patient _____

Does study partner live with patient? _____

If 'No' how many hours contact do they have a month _____

Years of F/T education _____

Patient's occupation _____ **Working/ retired** _____

Marital status _____

Ethnic Group _____

Smoking History:

☐ Never smoked
☐ Current smoker _____
☐ Ex-smoker (how many years since stopping) _____

Alcohol History:

No. of units per week _____

Previous history (no. of units per week) _____

AD history:

Date of onset of symptoms_____

Date of diagnosis _____

Family history of AD/Dementia _____

_____

History of infections over the past 5 years: _____

_____

IMLOC
Version: 1.0
Date: 21.07.2016

IMLOC: Past medical history CRF

IMLOC	
Subject No.	
Pt. initials	

Past and Existing Medical Conditions	Date Started	Date ended/ ongoing

Concomitant Medications CRF

IMLOC	
Subject No.	
Pt. initials	

Concomitant medication use 3 months prior to screen (including over the counter medications and current/previous hormone replacement therapy)

Medication name	Indication	Dose	Freq.	Route	Date Started	Ongoing/ Date Stopped

IMLOC	
Subject No.	
Pt. initials	
Date	
Assessor initials	

Physiological Measures

Height (cm) _____ **Weight (kg)** _____ **BMI**_____

ECG Time_____
Comments _____
_____

BP (after 3 min supine) _____ **Heart rate**_____

Respiration rate_____ **Temp** _____

BP (on standing) _____ **Heart rate** _____

BP (standing +3mins)_____ **Heart rate** _____

Mental Arithmetic:
Rest for 3 mins, BP_____ **Serial subtraction BP** _____

Cold pressure test:
BP _____ **Heart rate** _____

Bloods Time _____

University Hospital Southampton NHS
NHS Foundation Trust

MRI Safety Questionnaire

Patient Name: ..... **Date of Birth:** .....

Address: .....

Hospital Number:.....**Height**.....**Weight**.....

Body Part to be scanned.....

PLEASE COMPLETE THE QUESTIONS ON THIS SIDE OF THE FORM
TELEPHONE US ON 023 8120 6588 IF YOU ANSWER YES TO ANY OF THE QUESTIONS ON THIS SIDE OF THE PAGE.
BRING THE FORM WITH YOU TO YOUR SCAN APPOINTMENT.

1	Have you had any type of electronic, mechanical or magnetic implant added to any part of your body? *For example neurostimulator, implanted drug pump, programmable shunt.* Please give details.	Y/N	
2	Have you had any operations or procedures in the last 6 weeks? Please give details.	Y/N	
3	Have you ever had any operations or procedures on your heart? *For example stents, valve replacement, closure devices.* Please give details.	Y/N	
4	Do you have/ have you ever had a cardiac pacemaker, defibrillator, cardiac monitor? Please give as much detail as possible. *For example make, model, when it was fitted, which hospital?*	Y/N	
5	Do you have any hearing aids or a cochlear implant?	Y/N	
6	Have you ever had any operations or procedures on your head, eyes or ears? Please give details.	Y/N	
7	Have you ever had any operations involving the use of metal implants? *For example coils, clips, filters, screws, metal plates, joint replacements.* Please give details.	Y/N	
8	Have you ever been asked to swallow a capsule camera, "pillcam" to investigate your bowel?	Y/N	
9	Have you EVER had any accidents where metal has gone into your eyes? (E.g. welding, metalwork or other accidents)	Y/N	
10	Have you ever had any metal fragments in any other part of your body?	Y/N	
11	Is there any possibility that you may be pregnant? OR are you breast-feeding?	Y/N	
12	Have you ever had an allergic reaction to a contrast injection for an MRI scan?	Y/N	

Name of patient:		DOB:	/ /	Name of examiner:		Date of test:	/ /

Standardised Mini-Mental State Examination (SMMSE)

Please see accompanying guide for directions for administration

Say: I am going to ask you some questions and give you some problems to solve. Please try to answer as best you can.

1. **(Allow 10 seconds for each reply). Say:**

- **a)** What year is this? (Accept exact answer only) ..... / 1
- **b)** What season is this? (During the last week of the old season or first week of a new season, accept either) ..... / 1
- **c)** What month is this? (On the first day of a new month or the last day of the previous month, accept either) ..... / 1
- **d)** What is today's date? (Accept previous or next date) ..... / 1
- **e)** What day of the week is this? (Accept exact answer only) ..... / 1

2. **(Allow 10 seconds for each reply). Say:**

- **a)** What country are we in? (Accept exact answer only) ..... / 1
- **b)** What county are we in? (Accept exact answer only) ..... / 1
- **c)** What city/town are we in? (Accept exact answer only) ..... / 1
- **d)** (At home) What is the street address of this house? (Accept street name and house number or equivalent in rural areas)
  (In facility) What is the name of this building? (Accept exact name of institution only) ..... / 1
- **e)** (At home) What room are we in? (Accept exact answer only)
  (In facility) What floor of the building are we on? (Accept exact answer only) ..... / 1

3. **Say:** I am going to name three objects. When I am finished, I want you to repeat them. Remember what they are because I am going to ask you to name them again in a few minutes (Say slowly at approximately one-second intervals).

Ball Car Man

For repeated use: Bell, jar, fan; bill, tar, can; bull, bar, pan

Say: Please repeat the three items for me. (Score one point for each correct reply on the first attempt) ..... / 3

Allow 20 seconds for reply; if the person did not repeat all three, repeat until they are learned or up to a maximum of five times. (But only score first attempt).

4. **Spell the word WORLD.** (You may help the person to spell the word correctly) **Say:** *Now spell it backwards please* (Allow 30 seconds; if the subject cannot spell World even with assistance, score 0) Refer to accompanying guide for scoring instructions (Score on reverse of this sheet) ..... / 5

5. **Say:** Now what were the three objects I asked you to remember? ..... / 3

(Score one point for each correct answer regardless of order; allow 10 seconds)

6. **Show wristwatch. Ask:** *What is this called?* ..... / 1

(Score one point for correct response; accept "wristwatch" or "watch"; do not accept "clock" or "time", etc.; allow 10 seconds)

7. **Show pencil. Ask:** *What is this called?* ..... / 1

(Score one point for correct response; accept "pencil" only; score 0 for pen; allow 10 seconds for reply)

8. **Say:** I would like you to repeat a phrase after me: *No ifs, ands, or buts* ..... / 1

(Allow 10 seconds for response. Score one point for a correct repetition. Must be exact, e.g. no ifs or buts, score 0)

9. **Say:** *Read the words on this page and then do what it says* ..... / 1

Then, **hand** the person the sheet with CLOSE YOUR EYES (score on reverse of this sheet) on it. If the subject just reads and does not close eyes, you may repeat: Read the words on this page and then do what it says, (a maximum of three times. See point No. 3 in Directions for Administration section of accompanying guide). Allow 10 seconds, score one point only if the subject closes eyes. The subject does not have to read aloud.

10. **Hand** the person a pencil and paper. **Say:** *Write any complete sentence on that piece of paper.* (Allow 30 seconds. Score one point. The sentence must make sense. Ignore spelling errors) ..... / 1

11. **Place** design (see reverse of this sheet), pencil, eraser and paper in front of the person. **Say:** *Copy this design please.* Allow multiple tries. Wait until the person is finished and hands it back. Score one point for a correctly copied diagram. The person must have drawn a four-sided figure between two five-sided figures. Maximum time: One minute ..... / 1

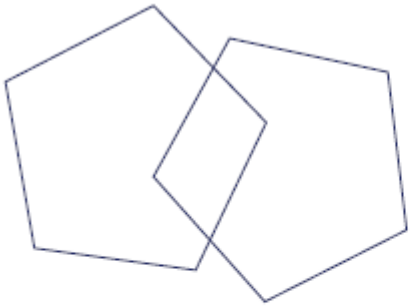
12. **Ask** the person if he is right or left handed. **Take** a piece of paper, hold it up in front of the person and **say** the following:
Take this paper in your right/left hand (whichever is non-dominant), fold the paper in half once with both hands and put the paper down on the floor.

Takes paper in correct hand ..... / 1
Folds it in half ..... / 1
Puts it on the floor ..... / 1

TOTAL TEST SCORE: / 30

ADJUSTED SCORE : /

Molloy DW, Alemayehu E, Roberts R. Reliability of a standardized Mini-Mental State Examination compared with the traditional Mini-Mental state Examination. *American Journal of Psychiatry*, Vol. 14, 1991a, pp.102-105.



Time: .....

D	L	R	O	W

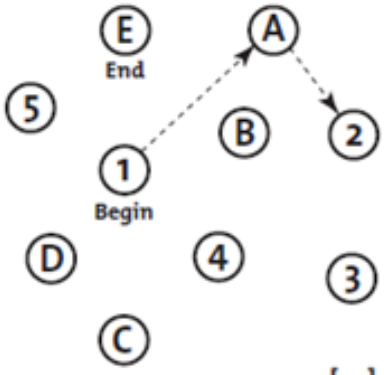
=

CLOSE YOUR EYES

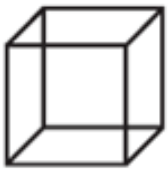
MONTREAL COGNITIVE ASSESSMENT (MOCA)

NAME :
Education : Date of birth :
Sex : DATE :

VISUOSPATIAL / EXECUTIVE



Copy cube



[ ] [ ]

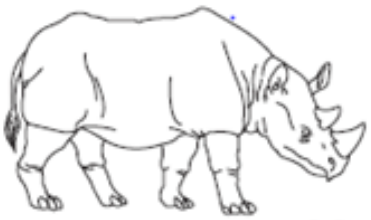
Draw CLOCK (Ten past eleven)
(3 points)

[ ] Contour [ ] Numbers [ ] Hands

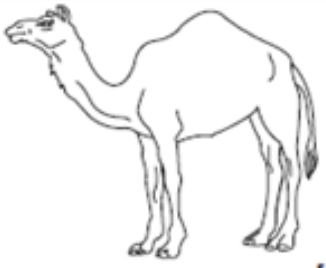
POINTS __/5

NAMING

[ ]



[ ]



[ ] __/3

MEMORY

Read list of words, subject must repeat them. Do 2 trials. Do a recall after 5 minutes.

	FACE	VELVET	CHURCH	DAISY	RED
1st trial					
2nd trial					

No points

ATTENTION

Read list of digits (1 digit/ sec.). Subject has to repeat them in the forward order [ ] 2 1 8 5 4

Subject has to repeat them in the backward order [ ] 7 4 2 __/2

Read list of letters. The subject must tap with his hand at each letter A. No points if ≥ 2 errors

[ ] F B A C M N A A J K L B A F A K D E A A A J A M O F A A B __/1

Serial 7 subtraction starting at 100 [ ] 93 [ ] 86 [ ] 79 [ ] 72 [ ] 65

4 or 5 correct subtractions: **3 pts**, 2 or 3 correct: **2 pts**, 1 correct: **1 pt**, 0 correct: **0 pt** __/3

LANGUAGE

Repeat : I only know that John is the one to help today. [ ]

The cat always hid under the couch when dogs were in the room. [ ] __/2

Fluency / Name maximum number of words in one minute that begin with the letter F [ ] _____ (N ≥ 11 words) __/1

ABSTRACTION

Similarity between e.g. banana - orange = fruit [ ] train – bicycle [ ] watch - ruler __/2

DELAYED RECALL

	Has to recall words WITH NO CUE	FACE	VELVET	CHURCH	DAISY	RED	Points for UNCUED recall only
		[ ]	[ ]	[ ]	[ ]	[ ]	
Optional	Category cue						
	Multiple choice cue						

__/5

ORIENTATION

[ ] Date [ ] Month [ ] Year [ ] Day [ ] Place [ ] City __/6

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Normal ≥ 26 / 30

TOTAL __/30

Add 1 point if ≤ 12 yr edu

Alzheimer's Disease Cooperative Study

ADAS – Cognitive Behavior

SAMPLE FORM – Page 1 of 4

Center Name	Patient Number	Patient Initials	Examiner Initials	Examination Date
	P R – –			Month Day Year

1. WORD RECALL TASK: Indicate the total number of ***correct*** responses for each trial

Trial 1	Trial 2	Trial 3

2. NAMING OBJECTS AND FINGERS: Check each object/finger named ***correctly*** or check "NONE."

NONE ☐

☐ Flower	☐ Rattle	☐ Wallet
☐ Bed	☐ Mask	☐ Harmonica
☐ Whistle	☐ Scissors	☐ Stethoscope
☐ Pencil	☐ Comb	☐ Tongs
☐ Thumb	☐ Index	☐ Ring
☐ Pinky	☐ Middle	

3. COMMANDS: Check each command performed ***correctly*** or check "NONE."

NONE ☐

- ☐ Make a fist.
- ☐ Point to the ceiling, then to the floor.
- ☐ Put the pencil on top of the card, then put it back.
- ☐ Put the watch on the other side of the pencil and turn over the card.
- ☐ Tap each shoulder twice with two fingers keeping your eyes shut.

4. CONSTRUCTIONAL PRAXIS: Check each figure drawn ***correctly***.

- ☐ None: attempted but drew no forms correctly.
- ☐ Patient drew no forms; scribbled; wrote words.
- ☐ Circle
- ☐ Two overlapping rectangles
- ☐ Rhombus
- ☐ Cube

5. IDEATIONAL PRAXIS: Check each step completed ***correctly*** or check "NONE"

NONE ☐

- ☐ Fold a letter.
- ☐ Put letter in envelope.
- ☐ Seal envelope.
- ☐ Address envelope.
- ☐ Indicate where stamp goes.

6. ORIENTATION: Check each item answered ***correctly*** or check "NONE."

NONE ☐

☐ Full name	☐ Day
☐ Month	☐ Season
☐ Date	☐ Place
☐ Year	☐ Time of day

7. WORD RECOGNITION TASK: Scoring will be done by the A.D.C.S. Data Coordinating Center.

Trial 1	Trial 2	Trial 3
X	X	X

8. LANGUAGE: Check level of impairment.

- ☐ **None**: patient speaks clearly and/or is understandable.
- ☐ **Very Mild**: one instance of lack of understandability.
- ☐ **Mild**: patient has difficulty < 25% of the time.
- ☐ **Moderate**: patient has difficulty 25–50% of the time.
- ☐ **Moderately Severe**: patient has difficulty more than 50% of the time.
- ☐ **Severe**: one- or two-word utterances; fluent, but empty speech; mute.

9. COMPREHENSION OF SPOKEN LANGUAGE: Check level of impairment

- ☐ **None**: patient understands.
- ☐ **Very Mild**: one instance of misunderstanding.
- ☐ **Mild**: 3–5 instances of misunderstanding.
- ☐ **Moderate**: requires several repetitions and rephrasing.
- ☐ **Moderately Severe**: patient only occasionally responds correctly; i.e., yes – no questions.
- ☐ **Severe**: patient rarely responds to questions appropriately; not due to poverty of speech.

10. WORD FINDING DIFFICULTY: Check one response.

- ☐ **None.**
- ☐ **Very Mild**: 1 or 2 instances, not clinically significant.
- ☐ **Mild**: noticeable circumlocution or synonym substitution.
- ☐ **Moderate**: loss of words without compensation on occasion.
- ☐ **Moderately Severe**: frequent loss of words without compensation.
- ☐ **Severe**: nearly total loss of content words; speech sounds empty; 1– to 2-word utterances.

11. REMEMBERING TEST INSTRUCTIONS: Check level of impairment.

- ☐ **None.**
- ☐ **Very Mild**: forgets once.
- ☐ **Mild**: must be reminded 2 times.
- ☐ **Moderate**: must be reminded 3–4 times.
- ☐ **Moderately Severe**: must be reminded 5–6 times
- ☐ **Severe**: must be reminded 7 or more times.

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Alzheimer's Disease Cooperative Study

ADAS – Word Recall

SAMPLE FORM – Page 2 of 4

Center Name	Patient Number	Patient Initials	Examiner Initials	Examination Date
	P R – –			Month Day Year

Present Word List #2.

Check EACH word correctly recalled.

TRIAL 1	
BOTTLE	
POTATO	
GIRL	
TEMPLE	
STAR	
ANIMAL	
FOREST	
LAKE	
CLOCK	
OFFICE	
TOTAL	

TRIAL 2	
FOREST	
TEMPLE	
BOTTLE	
STAR	
POTATO	
GIRL	
CLOCK	
ANIMAL	
LAKE	
OFFICE	
TOTAL	

TRIAL 3	
GIRL	
TEMPLE	
POTATO	
ANIMAL	
FOREST	
LAKE	
OFFICE	
CLOCK	
BOTTLE	
STAR	
TOTAL	

Indicate total number of words correctly recalled for EACH trial on the ADAS Cognitive Behavior Form.

12. Executive Function (Maze):

a. ☐☐ number of errors

b. ☐☐ time at completion or second error (total seconds)

13. Number Cancellation:

a. ☐☐ number of targets hit (Range: 0 - 40)

b. ☐☐ number of errors

c. ☐☐ number of times to remind of task

If any item(s) 1-13 are incomplete or not done, please specify reason:

- ☐ Subject too cognitively impaired to complete
- ☐ Subject was unable to complete for physical reasons
- ☐ Subject refused
- ☐ Not Done, for reason other than above explain: _____

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Alzheimer's Disease Cooperative Study

ADAS – Delayed Recall

SAMPLE FORM – Page 3 of 4

Center Name	Patient Number	Patient Initials	Examiner Initials	Examination Date
	P R – –			Month Day Year

Instructions: Say to the patient, **"NOW I WANT YOU TO TRY TO REMEMBER THE WORDS THAT I SHOWED YOU EARLIER ON PRINTED CARDS. CAN YOU TELL ME ANY OF THOSE WORDS?"**

Allow a maximum of two minutes for recall.

check EACH word correctly recalled.

BOTTLE	
POTATO	
GIRL	
TEMPLE	
STAR	
ANIMAL	
FOREST	
LAKE	
CLOCK	
OFFICE	
TOTAL	

WHITE- ADCS COPY YELLOW- INVESTIGATOR'S COPY PINK- CLINICAL MONITOR'S COPY

Alzheimer's Disease Cooperative Study

ADAS – Word Recognition
SAMPLE FORM – Page 4 of 4

Center Name	Patient Number	Patient Initials	Examiner Initials	Examination Date
	P R – –			Month Day Year

Present Word List #2.

Check subject's response for each word. Subject should respond "yes" to original words which are bolded. INCORRECT responses are shaded. Three trials of reading and recognition are given.

	Yes	No
COST		
NATION		
CHIMNEY		
SPARROW		
DAMAGES		
TRAFFIC		
SANDWICH		
SERVICE		
SHELL		
SOLUTION		
YARD		
TUBE		
BODY		
GROUND		
STICK		
ENGINE		
RICHES		
GRAVITY		
SUMMER		
WISDOM		
MAN		
MEAL		
PASSENGER		
ACID		

	Yes	No
BATTLE		
MUCH		
TUBE		
TEAM		
COPY		
ENGINE		
GRAVITY		
COST		
JAR		
DISTANCE		
TRIUMPH		
TEMPER		
SENTENCE		
FOX		
PASSENGER		
SANDWICH		
SOLUTION		
WHISTLE		
CHIMNEY		
UNION		
ACID		
MEAL		
DAMAGES		
RICHES		

	Yes	No
VISITOR		
ACID		
SPEAK		
SOLUTION		
NAME		
MEAL		
LINE		
BILL		
CHIMNEY		
ENGINE		
WEALTH		
TUBE		
IMAGE		
COST		
SANDWICH		
DAMAGES		
ELEPHANT		
RICHES		
GRAVITY		
FUTURE		
PASSENGER		
STRING		
BANNER		
BERRY		

*see procedures manual for further clarification

WHITE- ADCS COPY YELLOW- INVESTIGATOR'S COPY PINK- CLINICAL MONITOR'S COPY

Subject's Initials_____ID#_____Date_____Time_____AM PM

PITTSBURGH SLEEP QUALITY INDEX

INSTRUCTIONS:
The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

1. During the past month, what time have you usually gone to bed at night?

   BED TIME _____

2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night?

   NUMBER OF MINUTES _____

3. During the past month, what time have you usually gotten up in the morning?

   GETTING UP TIME _____

4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed.)

   HOURS OF SLEEP PER NIGHT _____

For each of the remaining questions, check the one best response. Please answer all questions.

5. During the past month, how often have you had trouble sleeping because you . . .

a) Cannot get to sleep within 30 minutes

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

b) Wake up in the middle of the night or early morning

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

c) Have to get up to use the bathroom

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

d) Cannot breathe comfortably

Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

e) Cough or snore loudly

Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

f) Feel too cold

Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

g) Feel too hot

Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

h) Had bad dreams

Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

i) Have pain

Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

j) Other reason(s), please describe_____

_____

How often during the past month have you had trouble sleeping because of this?

Not during the past month_____ Less than once a week_____ Once or twice a week_____ Three or more times a week_____

6. During the past month, how would you rate your sleep quality overall?

Very good _____

Fairly good _____

Fairly bad _____

Very bad _____

7. During the past month, how often have you taken medicine to help you sleep (prescribed or "over the counter")?

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

9. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?

No problem at all _____

Only a very slight problem _____

Somewhat of a problem _____

A very big problem _____

10. Do you have a bed partner or room mate?

No bed partner or room mate _____

Partner/room mate in other room _____

Partner in same room, but not same bed _____

Partner in same bed _____

If you have a room mate or bed partner, ask him/her how often in the past month you have had . . .

a) Loud snoring

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

b) Long pauses between breaths while asleep

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

c) Legs twitching or jerking while you sleep

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

d) Episodes of disorientation or confusion during sleep

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

e) Other restlessness while you sleep; please describe_____

_____

Not during the past month_____	Less than once a week_____	Once or twice a week_____	Three or more times a week_____

Buysse DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ: Psychiatry Research, 28:193-213, 1989.

Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

Information obtained through: ☐ Informant visit ☐ Telephone call

Instructions: For each question, use the subject's name where (s) appears. Before beginning, read the questionnaire guidelines to the informant.

1. Regarding **eating**:

Which best describes (S) usual performance during the past 4 weeks?

3 ☐ ate without physical help, and used a knife
2 ☐ used a fork or spoon, but not a knife, to eat
1 ☐ used fingers to eat
0 ☐ (S) usually or always was fed by someone else

2. Regarding **walking** (or getting around in a wheelchair), in the past 4 weeks, which best describes his/her **optimal** performance:

3 ☐ mobile outside of home without physical help
2 ☐ mobile across a room without physical help
1 ☐ transferred from bed to chair without help
0 ☐ required physical help to walk or transfer

3. Regarding bowel and bladder function **at the toilet,** which best describes his/her **usual** performance in the past 4 weeks:

3 ☐ did everything necessary without supervision or help
2 ☐ needed supervision, but no physical help
1 ☐ needed physical help, and was usually continent
0 ☐ needed physical help, and was usually incontinent

Galasko, D., Bennett, D., Sano, M., Ernesto, E., Thomas, R., Grundman, M., and Ferris, S. *Alzheimer Disease and Associated Disorders* 1997; 11:S33-S39.

Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

4. Regarding **bathing,** in the past 4 weeks, which best describes his/her **usual** performance:

- 3 ☐ bathed without reminding or physical help
- 2 ☐ no physical help, but needed supervision/reminders to bathe completely
- 1 ☐ needed minor physical help (e.g., with washing hair) to bathe completely
- 0 ☐ needed to be bathed completely

5. Regarding **grooming,** in the past 4 weeks, which best describes his/her **optimal** performance:

- 3 ☐ cleaned and cut fingernails without physical help
- 2 ☐ brushed or combed hair without physical help
- 1 ☐ kept face and hands clean without physical help
- 0 ☐ needed help for grooming of hair, face, hands, and fingernails

Yes	No	Don't Know
☐	☐	☐
	0	0

6. Regarding **dressing, in the past 4 weeks:**

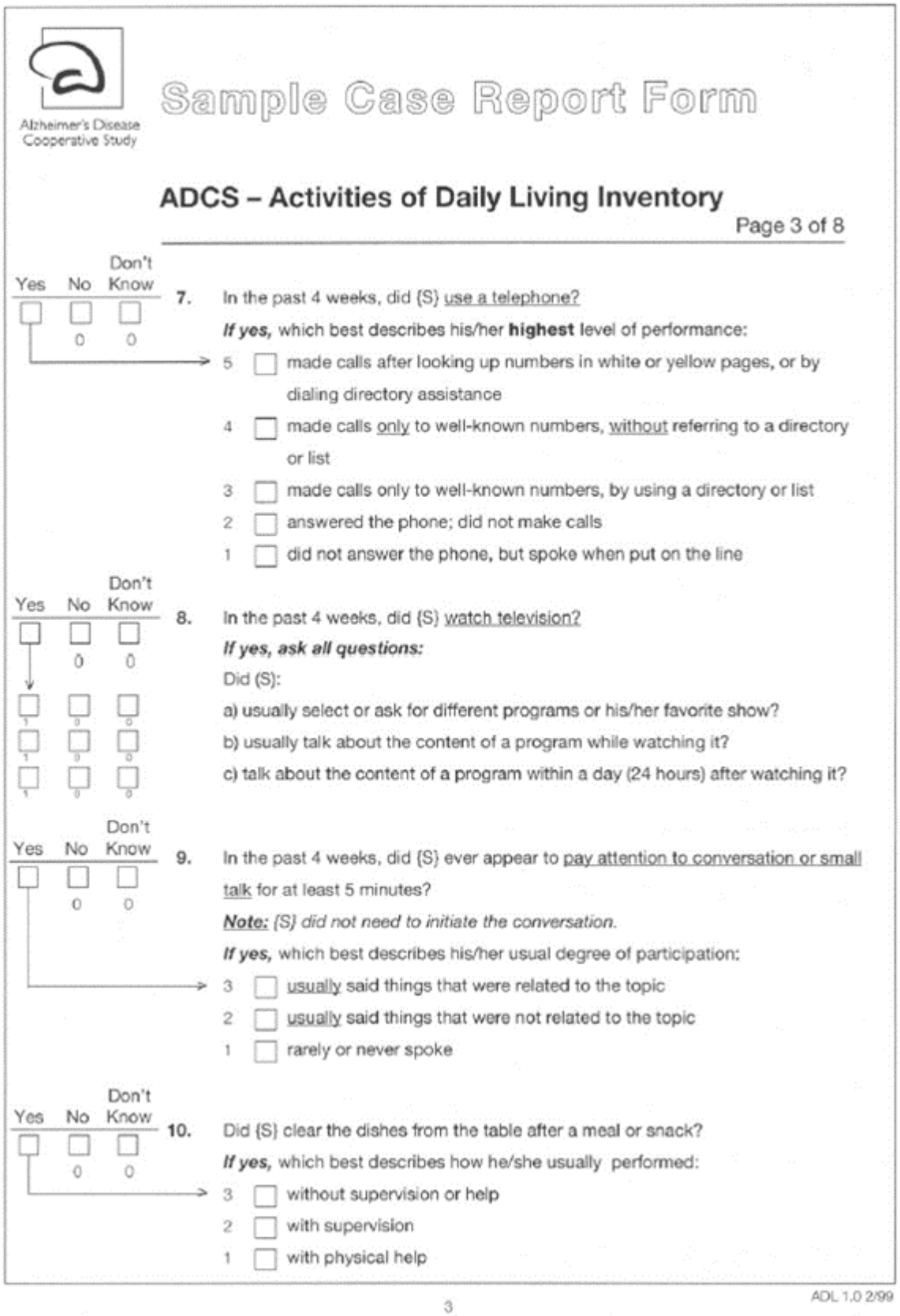
A) Did {S} select his/her first set of clothes for the day?

If yes, which best describes his/her usual performance:

- → 3 ☐ without supervision or help
- 2 ☐ with supervision
- 1 ☐ with physical help

B) Regarding physically getting dressed, which best describes his/her usual performance in the past 4 weeks:

- 4 ☐ dressed completely without supervision or physical help
- 3 ☐ dressed completely with supervision, but without help
- 2 ☐ needed physical help only for buttons, clasps, or shoelaces
- 1 ☐ dressed without help if clothes needed no fastening or buttoning
- 0 ☐ always needed help, regardless of the type of clothing



Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

Yes (0) | No | Don't Know (0)

7. In the past 4 weeks, did {S} use a telephone?

If yes, which best describes his/her **highest** level of performance:

5 ☐ made calls after looking up numbers in white or yellow pages, or by dialing directory assistance

4 ☐ made calls only to well-known numbers, without referring to a directory or list

3 ☐ made calls only to well-known numbers, by using a directory or list

2 ☐ answered the phone; did not make calls

1 ☐ did not answer the phone, but spoke when put on the line

Yes | No (0) | Don't Know (0)

8. In the past 4 weeks, did {S} watch television?

If yes, ask all questions:

Did (S):

a) usually select or ask for different programs or his/her favorite show? (1 | 0 | 0)

b) usually talk about the content of a program while watching it? (1 | 0 | 0)

c) talk about the content of a program within a day (24 hours) after watching it? (1 | 0 | 0)

Yes | No (0) | Don't Know (0)

9. In the past 4 weeks, did {S} ever appear to pay attention to conversation or small talk for at least 5 minutes?

Note: *{S) did not need to initiate the conversation.*

If yes, which best describes his/her usual degree of participation:

3 ☐ usually said things that were related to the topic

2 ☐ usually said things that were not related to the topic

1 ☐ rarely or never spoke

Yes | No (0) | Don't Know (0)

10. Did {S} clear the dishes from the table after a meal or snack?

If yes, which best describes how he/she usually performed:

3 ☐ without supervision or help

2 ☐ with supervision

1 ☐ with physical help

Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

Yes | No | Don't Know

11. In the past 4 weeks, did {S} usually manage to find his/her personal belongings at home?

If yes, which best describes how he/she usually performed:

- 3 ☐ without supervision or help
- 2 ☐ with supervision
- 1 ☐ with physical help

Yes | No | Don't Know

12. In the past 4 weeks, did {S} obtain a hot or cold beverage for him/herself? (A cold beverage includes a glass of water.)

If yes, which describes his/her highest level of performance

- 3 ☐ made a hot beverage, usually without physical help
- 2 ☐ made a hot beverage, usually if someone else heated the water
- 1 ☐ obtained a cold beverage, usually without physical help

Yes | No | Don't Know

13. In the past 4 weeks, did {S} make him/herself a meal or snack at home?

If yes, which best describes his/her highest level of food preparation:

- 4 ☐ cooked or microwaved food, with little or no help
- 3 ☐ cooked or microwaved food, with extensive help
- 2 ☐ mixed or combined food items for a meal or snack, without cooking or microwaving (e.g., made a sandwich)
- 1 ☐ obtained food on his/her own, without mixing or cooking it

Yes | No | Don't Know

14. In the past 4 weeks, did {S} dispose of garbage or litter in an appropriate place or container at home?

If yes, which best describes how he/she usually performed:

- 3 ☐ without supervision or help
- 2 ☐ with supervision
- 1 ☐ with physical help

Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

Yes ☐ 1 | No ☐ 0 | Don't Know ☐ 0 **15.** In the past 4 weeks, did {S} get around (or travel) outside of his/her home?

If yes, which best describes his/her **optimal** performance:

4 ☐ alone, went at least 1 mile away from home
3 ☐ alone, but remained within 1 mile of home
2 ☐ only when accompanied and supervised, regardless of the trip
1 ☐ only with physical help, regardless of the trip

Yes ☐ 1 | No ☐ 0 | Don't Know ☐ 0 **16.** In the past 4 weeks, did {S} ever go shopping ?

If yes, ask A and B:

A) Which one best describes how {S} usually selects items:

3 ☐ without supervision or physical help?
2 ☐ with some supervision or physical help?
1 ☐ not at all, or selected mainly random or inappropriate items?

B) Did {S} usually pay for items without supervision or physical help?

☐ 1 | ☐ 0 | ☐ 0

Yes ☐ 1 | No ☐ 0 | Don't Know ☐ 0 **17.** In the past 4 weeks, did {S} keep appointments or meetings with other people, such as relatives, a doctor, the hairdresser, etc.?

If yes, which best describes his/her awareness of the meeting ahead of time:

3 ☐ usually remembered, may have needed written reminders
e.g. notes, a diary, or calendar
2 ☐ only remembered the appointment after verbal reminders on the day
1 ☐ usually did not remember, in spite of verbal reminders on the day

Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

Note: 1. Do not ask Q18 if the {S} is institutionalized; check here ☐
2. Being taken to day care or having a sitter at home does not constitute being left alone.

Yes	No	Don't Know		
☐	☐ 0	☐ 0	**18.**	In the past 4 weeks, was {S} ever left on his/her own ? ***If yes, ask all questions:*** Was {S} left:
☐ 1	☐ 0	☐ 0		a) away from home, for 15 minutes or longer, during the day?
☐ 1	☐ 0	☐ 0		b) at home, for an hour or longer, during the day?
☐ 1	☐ 0	☐ 0		c) at home, for less than 1 hour, during the day?

Yes	No	Don't Know		
☐	☐ 0	☐ 0	**19.**	In the past 4 weeks, did {S} talk about **current** events? (This means events or incidents that occurred during the past month.) ***If yes, ask all questions:*** Did {S} talk about events that…:
☐ 1	☐ 0	☐ 0		a) he/she heard or read about or saw on TV but did not take part in?
☐ 1	☐ 0	☐ 0		b) he/she took part in outside home involving family, friends, or neighbors?
☐ 1	☐ 0	☐ 0		c) events that occurred at home that he/she took part in or watched?

Yes	No	Don't Know		
☐	☐ 0	☐ 0	**20.**	In the past 4 weeks, did {S} read a magazine, newspaper or book for more than 5 minutes at a time? ***If yes, ask all questions:*** Did {S} usually:
☐ 1	☐ 0	☐ 0		a) talk about details of what he/she read while or shortly (< than 1 hour) after reading?
☐ 1	☐ 0	☐ 0		b) talk about what he/she read 1 hour or longer after reading?

Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

Yes | No | Don't Know

21. In the past 4 weeks, did {S} ever write things down?

☐ | ☐ 0 | ☐ 0

Note: *If {S} wrote things only after encouragement or with help, the response should still be 'yes.'*

If yes, which best describes the most complicated things that he/she wrote:

3 ☐ letters or long notes that other people understood
2 ☐ short notes or messages that other people understood
1 ☐ his/her signature or name

Yes | No | Don't Know

22. In the past 4 weeks, did {S} perform a pastime, hobby or game?

☐ | ☐ 0 | ☐ 0

If yes, which pastimes did he/she perform:

Ask about all of the following, check all that apply:

☐ card or board games (including bridge, chess, checkers)
☐ bingo ☐ crosswords ☐ art
☐ musical instrument ☐ knitting ☐ sewing
☐ reading ☐ gardening ☐ golf
☐ tennis ☐ workshop ☐ fishing
☐ other _____

Note: *Walking does NOT count as a hobby/pastime for this scale.*

☐ **If {S} performs hobbies/pastimes only at day care, check here.**

If yes, how did {S} usually perform his/her most common pastimes:

3 ☐ without supervision or help
2 ☐ with supervision
1 ☐ with help

ADL 1.0 2/99

Alzheimer's Disease Cooperative Study

Sample Case Report Form

ADCS – Activities of Daily Living Inventory

Yes	No	Don't Know
☐	☐	☐
	0	0

23. In the past 4 weeks, did {S} use a household appliance to do chores?

→ ***Ask about all of the following,*** and check those that were used:

- ☐ washer
- ☐ dryer
- ☐ vacuum
- ☐ dishwasher
- ☐ toaster
- ☐ toaster oven
- ☐ range
- ☐ microwave
- ☐ food processor
- ☐ other _____

If yes, for the most commonly used appliances, which best describes how {S} usually used them:

4 ☐ without help, operating more than on-off controls if needed
3 ☐ without help, but operated only on/off controls
2 ☐ with supervision, but no physical help
1 ☐ with physical help

☐☐ Total Score (0-78)

☐☐ Number of "Don't Know" Responses

A. Delusions (NA)

Does the patient have beliefs that you know are not true (for example, insisting that people are trying to harm him/her or steal from him/her)? Has he/she said that family members are not who they say they are or that the house is not their home? I'm not asking about mere suspiciousness; I am interested if the patient is convinced that these things are happening to him/her.

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient believe that he/she is in danger - that others are planning to hurt him/her? _____
2. Does the patient believe that others are stealing from him/her? _____
3. Does the patient believe that his/her spouse is having an affair? _____
4. Does the patient believe that unwelcome guests are living in his/her house? _____
5. Does the patient believe that his/her spouse or others are not who they claim to be? _____
6. Does the patient believe that his/her house is not his/her home? _____
7. Does the patient believe that family members plan to abandon him/her? _____
8. Does the patient believe that television or magazine figures are actually present in the home? [Does he/she try to talk or interact with them?] _____
9. Does the patient believe any other unusual things that I haven't asked about? _____

If the screening question is confirmed, determine the frequency and severity of the delusions.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - once or more per day.

Severity:
1. Mild - delusions present but seem harmless and produce little distress in the patient.
2. Moderate - delusions are distressing and disruptive.
3. Marked - delusions are very disruptive and are a major source of behavioral disruption. [If PRN medications are prescribed, their use signals that the delusions are of marked severity.]

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

B. Hallucinations (NA)

Does the patient have hallucinations such as seeing false visions or hearing imaginary voices? Does he/she seem to see, hear or experience things that are not present? By this question we do not mean just mistaken beliefs such as stating that someone who has died is still alive; rather we are asking if the patient actually has abnormal experiences of sounds or visions.

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient describe hearing voices or act as if he/she hears voices? _____
2. Does the patient talk to people who are not there? _____
3. Does the patient describe seeing things not seen by others or behave as if he/she is seeing things not seen by others (people, animals, lights, etc)? _____
4. Does the patient report smelling odors not smelled by others? _____
5. Does the patient describe feeling things on his/her skin or otherwise appear to be feeling things crawling or touching him/her? _____
6. Does the patient describe tastes that are without any known cause? _____
7. Does the patient describe any other unusual sensory experiences? _____

If the screening question is confirmed, determine the frequency and severity of the hallucinations.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - once or more per day.

Severity:
1. Mild - hallucinations are present but harmless and cause little distress for the patient.
2. Moderate - hallucinations are distressing and are disruptive to the patient.
3. Marked - hallucinations are very disruptive and are a major source of behavioral disturbance. PRN medications may be required to control them.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

C. Agitation/Aggression **(NA)**

Does the patient have periods when he/she refuses to cooperate or won't let people help him/her? Is he/she hard to handle?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient get upset with those trying to care for him/her or resist activities such as bathing or changing clothes? _____
2. Is the patient stubborn, having to have things his/her way? _____
3. Is the patient uncooperative, resistive to help from others? _____
4. Does the patient have any other behaviors that make him hard to handle? _____
5. Does the patient shout or curse angrily? _____
6. Does the patient slam doors, kick furniture, throw things? _____
7. Does the patient attempt to hurt or hit others? _____
8. Does the patient have any other aggressive or agitated behaviors? _____

If the screening question is confirmed, determine the frequency and severity of the agitation.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than daily.
4. Very frequently - once or more per day.

Severity:
1. Mild - behavior is disruptive but can be managed with redirection or reassurance.
2. Moderate - behaviors are disruptive and difficult to redirect or control.
3. Marked - agitation is very disruptive and a major source of difficulty; there may be a threat of personal harm. Medications are often required.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

D. Depression/Dysphoria **(NA)**

Does the patient seem sad or depressed? Does he/she say that he/she feels sad or depressed?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient have periods of tearfulness or sobbing that seem to indicate sadness? _____
2. Does the patient say or act as if he/she is sad or in low spirits? _____
3. Does the patient put him/herself down or say that he/she feels like a failure? _____
4. Does the patient say that he/she is a bad person or deserves to be punished? _____
5. Does the patient seem very discouraged or say that he/she has no future? _____
6. Does the patient say he/she is a burden to the family or that the family would be better off without him/her? _____
7. Does the patient express a wish for death or talk about killing him/herself? _____
8. Does the patient show any other signs of depression or sadness? _____

If the screening question is confirmed, determine the frequency and severity of the depression.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - essentially continuously present.

Severity:
1. Mild - depression is distressing but usually responds to redirection or reassurance.
2. Moderate - depression is distressing, depressive symptoms are spontaneously voiced by the patient and difficult to alleviate.
3. Marked - depression is very distressing and a major source of suffering for the patient.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

E. Anxiety (NA)

Is the patient very nervous, worried, or frightened for no apparent reason? Does he/she seem very tense or fidgety? Is the patient afraid to be apart from you?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient say that he/she is worried about planned events? _____
2. Does the patient have periods of feeling shaky, unable to relax, or feeling excessively tense? _____
3. Does the patient have periods of [or complain of] shortness of breath, gasping, or sighing for no apparent reason other than nervousness? _____
4. Does the patient complain of butterflies in his/her stomach, or of racing or pounding of the heart in association with nervousness? [Symptoms not explained by ill health] _____
5. Does the patient avoid certain places or situations that make him/her more nervous such as riding in the car, meeting with friends, or being in crowds? _____
6. Does the patient become nervous and upset when separated from you [or his/her caregiver]? [Does he/she cling to you to keep from being separated?] _____
7. Does the patient show any other signs of anxiety? _____

If the screening question is confirmed, determine the frequency and severity of the anxiety.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - once or more per day.

Severity:
1. Mild - anxiety is distressing but usually responds to redirection or reassurance.
2. Moderate - anxiety is distressing, anxiety symptoms are spontaneously voiced by the patient and difficult to alleviate.
3. Marked - anxiety is very distressing and a major source of suffering for the patient.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

F. Elation/Euphoria **(NA)**

Does the patient seem too cheerful or too happy for no reason? I don't mean the normal happiness that comes from seeing friends, receiving presents, or spending time with family members. I am asking if the patient has a persistent and abnormally good mood or finds humor where others do not.

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient appear to feel too good or to be too happy, different from his/her usual self? _____
2. Does the patient find humor and laugh at things that others do not find funny? _____
3. Does the patient seem to have a childish sense of humor with a tendency to giggle or laugh inappropriately (such as when something unfortunate happens to others)? _____
4. Does the patient tell jokes or make remarks that have little humor for others but seem funny to him/her? _____
5. Does he/she play childish pranks such as pinching or playing "keep away" for the fun of it? _____
6. Does the patient "talk big" or claim to have more abilities or wealth than is true? _____
7. Does the patient show any other signs of feeling too good or being too happy? _____

If the screening question is confirmed, determine the frequency and severity of the elation/euphoria.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - essentially continuously present.

Severity:
1. Mild - elation is notable to friends and family but is not disruptive.
2. Moderate - elation is notably abnormal.
3. Marked - elation is very pronounced; patient is euphoric and finds nearly everything to be humorous.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

G. Apathy/Indifference **(NA)**

Has the patient lost interest in the world around him/her? Has he/she lost interest in doing things or does he/she lack motivation for starting new activities? Is he/she more difficult to engage in conversation or in doing chores? Is the patient apathetic or indifferent?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient seem less spontaneous and less active than usual? _____
2. Is the patient less likely to initiate a conversation? _____
3. Is the patient less affectionate or lacking in emotions when compared to his/her usual self? _____
4. Does the patient contribute less to household chores? _____
5. Does the patient seem less interested in the activities and plans of others? _____
6. Has the patient lost interest in friends and family members? _____
7. Is the patient less enthusiastic about his/her usual interests? _____
8. Does the patient show any other signs that he/she doesn't care about doing new things? _____

If the screening question is confirmed, determine the frequency and severity of the apathy/indifference.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - nearly always present.

Severity:
1. Mild - apathy is notable but produces little interference with daily routines; only mildly different from patient's usual behavior; patient responds to suggestions to engage in activities.
2. Moderate - apathy is very evident; may be overcome by the caregiver with coaxing and encouragement; responds spontaneously only to powerful events such as visits from close relatives or family members.
3. Marked - apathy is very evident and usually fails to respond to any encouragement or external events.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

H. Disinhibition **(NA)**

Does the patient seem to act impulsively without thinking? Does he/she do or say things that are not usually done or said in public? Does he/she do things that are embarrassing to you or others?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient act impulsively without appearing to consider the consequences? _____
2. Does the patient talk to total strangers as if he/she knew them? _____
3. Does the patient say things to people that are insensitive or hurt their feelings? _____
4. Does the patient say crude things or make sexual remarks that he/she would not usually have said? _____
5. Does the patient talk openly about very personal or private matters not usually discussed in public? _____
6. Does the patient take liberties or touch or hug others in way that is out of character for him/her? _____
7. Does the patient show any other signs of loss of control of his/her impulses? _____

If the screening question is confirmed, determine the frequency and severity of the disinhibition.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - essentially continuously present.

Severity:
1. Mild - disinhibition is notable but usually responds to redirection and guidance.
2. Moderate - disinhibition is very evident and difficult to overcome by the caregiver.
3. Marked - disinhibition usually fails to respond to any intervention by the caregiver, and is a source of embarrassment or social distress.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

I. Irritability/Lability (NA)

Does the patient get irritated and easily disturbed? Are his/her moods very changeable? Is he/she abnormally impatient? We do not mean frustration over memory loss or inability to perform usual tasks; we are interested to know if the patient has abnormal irritability, impatience, or rapid emotional changes different from his/her usual self.

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient have a bad temper, flying "off the handle" easily over little things? _____
2. Does the patient rapidly change moods from one to another, being fine one minute and angry the next? _____
3. Does the patient have sudden flashes of anger? _____
4. Is the patient impatient, having trouble coping with delays or waiting for planned activities? _____
5. Is the patient cranky and irritable? _____
6. Is the patient argumentative and difficult to get along with? _____
7. Does the patient show any other signs of irritability? _____

If the screening question is confirmed, determine the frequency and severity of the irritability/lability.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - essentially continuously present.

Severity:
1. Mild - irritability or lability is notable but usually responds to redirection and reassurance.
2. Moderate - irritability and lability are very evident and difficult to overcome by the caregiver.
3. Marked - irritability and lability are very evident, they usually fail to respond to any intervention by the caregiver, and they are a major source of distress.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

J. Aberrant Motor Behavior **(NA)**

Does the patient pace, do things over and over such as opening closets or drawers, or repeatedly pick at things or wind string or threads?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient pace around the house without apparent purpose? _____
2. Does the patient rummage around opening and unpacking drawers or closets? _____
3. Does the patient repeatedly put on and take off clothing? _____
4. Does the patient have repetitive activities or "habits" that he/she performs over and over? _____
5. Does the patient engage in repetitive activities such as handling buttons, picking, wrapping string, etc? _____
6. Does the patient fidget excessively, seem unable to sit still, or bounce his/her feet or tap his/her fingers a lot? _____
7. Does the patient do any other activities over and over? _____

If the screening question is confirmed, determine the frequency and severity of the aberrant motor activity:

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - essentially continuously present.

Severity:
1. Mild - abnormal motor activity is notable but produces little interference with daily routines.
2. Moderate - abnormal motor activity is very evident; can be overcome by the caregiver.
3. Marked - abnormal motor activity is very evident, usually fails to respond to any intervention by the caregiver, and is a major source of distress.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

K. Sleep (NA)

Does the patient have difficulty sleeping (do not count as present if the patient simply gets up once or twice per night only to go to the bathroom and falls back asleep immediately)? Is he/she up at night? Does he/she wander at night, get dressed, or disturb your sleep?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Does the patient have difficulty falling asleep? _____
2. Does the patient get up during the night (do not count if the patient gets up once or twice per night only to go to the bathroom and falls back asleep immediately)? _____
3. Does the patient wander, pace, or get involved in inappropriate activities at night? _____
4. Does the patient awaken you during the night? _____
5. Does the patient awaken at night, dress, and plan to go out thinking that it is morning and time to start the day? _____
6. Does the patient awaken too early in the morning (earlier that was his/her habit)? _____
7. Does the patient sleep excessively during the day? _____
8. Does the patient have any other nighttime behaviors that bother you that we haven't talked about? _____

If the screening question is confirmed, determine the frequency and severity of the nighttime behavior disturbance.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - once or more per day (every night)

Severity:
1. Mild - nighttime behaviors occur but they are not particularly disruptive.
2. Moderate - nighttime behaviors occur and disturb the patient and the sleep of the caregiver; more than one type of nighttime behavior may be present.
3. Marked - nighttime behaviors occur; several types of nighttime behavior may be present; the patient is very distressed during the night and the caregiver's sleep is markedly disturbed.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

L. Appetite and eating disorders **(NA)**

Has he/she had any change in appetite, weight, or eating habits (count as NA if the patient is incapacitated and has to be fed)? Has there been any change in type of food he/she prefers?

NO (If no, proceed to next screening question). YES (If yes, proceed to subquestions).

1. Has he/she had a loss of appetite? _____
2. Has he/she had an increase in appetite? _____
3. Has he/she had a loss of weight? _____
4. Has he/she gained weight? _____
5. Has he/she had a change in eating behavior such as putting too much food in his/her mouth at once? _____
6. Has he/she had a change in the kind of food he/she likes such as eating too many sweets or other specific types of food? _____
7. Has he/she developed eating behaviors such as eating exactly the same types of food each day or eating the food in exactly the same order? _____
8. Have there been any other changes in appetite or eating that I haven't asked about? _____

If the screening question is confirmed, determine the frequency and severity of the changes in eating habits or appetite.

Frequency:
1. Occasionally - less than once per week.
2. Often - about once per week.
3. Frequently - several times per week but less than every day.
4. Very frequently - once or more per day or continuously

Severity:
1. Mild - changes in appetite or eating are present but have not led to changes in weight and are not disturbing
2. Moderate - changes in appetite or eating are present and cause minor fluctuations in weight.
3. Marked - obvious changes in appetite or eating are present and cause fluctuations in weight, are embarrassing, or otherwise disturb the patient.

Distress: How emotionally distressing do you find this behavior?
0. Not at all
1. Minimally
2. Mildly
3. Moderately
4. Severely
5. Very severely or extremely

Appendix D Immunohistochemistry

1) Rehydrate slides.
   Clearene 1: 10 min
   Clearene 2: 10 min
   100% Ethanol: 5 min
   100% Ethanol: 5min
   70% Ethanol: 5min
   Tap water
   Distilled water

2) Bleach neuromelanin pigment and block endogenous peroxidase: with 10% H_2O_2 overnight for 20 hours (make from 30% stock, 1:2 ratio with TBS i.e. 1ml of 30% H_2O_2 and 2ml TBS).

3) TBS wash: 3 x 10 secs RT *(defrost blocking solution for step 6)*

4) Antigen Retrieval (method depends on primary antibody used, see Table 10 either citrate or EDTA buffer 50% or 100% power for 25mins)
   - Citrate buffer: 2.1g citric acid-monoxydrate in 1L distilled H2O, pH to 6 with ~25ml of 1M NaOH, microwave with slides
   - EDTA buffer: 0.37g EDTA in 1L distilled H2O, pH to 8 with ~8ml of 0.1M NaOH, microwave with slides

   Cool down with tap water for 1-2 mins

5) TBS wash: 3 x 10 secs RT

6) Blocking solution to remove non-specific binding 20 mins RT (depends on host used for production of secondary antibodies)
   - Swine anti-rabbit (Dako) blocking medium contains DMEM, foetal calf serum and bovine albumin
   - Goat anti-mouse (Vector), 5% normal goat serum
   - Rabbit anti-goat (Dako), 5% normal rabbit serum

7) **Error! Reference source not found.**)

8) TBS wash: 3 x 10 secs RT

9) Secondary Antibody from the host species of the primary antibody conjugated with biotin 30 mins RT
   - Biotinylated goat anti-mouse Vector 1:800
   - Biotinylated swine anti-rabbit Dako 1:800
   - Biotinylated rabbit anti-goat Dako

10) TBS wash: 3 x 10 secs RT

11) ABC-HRP Avidin/biotin complex. Avidin, labelled with peroxidase, has a high affinity for biotin therefore amplifying the signal from the secondary antibody 30 mins RT (A 1/75, B 1/75 and TBS e.g. 5.3µl A, 5.3µl B, 400µl TBS)

12) TBS wash: 3 x 10 secs RT

13) 3'3- Diaminobenzidine (DAB) for chromogenic reaction, oxidase with the peroxidase to form a brown precipitate (2 drops buffer, 2 drops H_2O_2, 4 drops DAB, 5ml distilled water) duration depends on tissue and antibody see Table 10.

14) Stop DAB reaction with TBS then distilled water then tap water for 5 mins

15) Counterstain with Haematoxylin 30 secs RT

16) Stop reaction with distilled water, then tap water for 5 mins

17) Dehydration:
    70% Ethanol: 1min
    100% Ethanol: 1 min
    100% Ethanol: 1 min
    Clearene 1: 3 min
    Clearene 2: 3 min
    Clearene 3: 3 min

18) Mount with XTF and cover

19) Clean slides once dry (best to leave overnight) for slide scanner

Reagents used

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 - H_2O_2 solution 30% in H20, H1009
Vectastain® ABC kit, PK6100
Vector - DAB peroxidase substrate kit, SK4100
Dako - antibody dilutent background reducing, S3022

Appendix E Post-mortem case details

Table 17. Details of the cases used in the post-mortem study, provided by SWDBB

Case ID	Braak stage	Age	Gender	Clinical Diagnosis	Histological Diagnosis	Frozen tissue
751	0	80	M	CONTROL/ COPD	Mod CAA, mild to mod cerebrovascular disease	Yes
851	0	68	F	CONTROL	Control, mild arteriosclerotic cerebrovascular disease, occipital CAA	Yes
1010	0	77	M	CONTROL	Mild diffuse plaque and Lewy body pathology	Yes
1068	0	86	M	CONTROL	Normal brain	Yes
803	1	77	M	CONTROL	No AD, mild to moderate CAA	Yes
881	1	86	F	CONTROL	Control, severe arteriosclerotic small vessel disease with microinfarcts	Yes
887	1	74	F	CONTROL	Control, no significant abnormalities	Yes
921	1	96	F	CONTROL	Moderate SVD, moderate CAA and extensive very acute cerebral infarction (involving F, Tp and P lobes)	Yes
941	1	92	M	CONTROL	Control, moderate CAA	Yes
971	1	90	F	CONTROL	Single microinfarct in occipital cortex - fine to use as control	Yes
1067	1	69	F	CONTROL	Normal brain	Yes
1083	1	94	F	CONTROL	Normal-looking brain	Yes
766	2	92	M	CONTROL	No AD, moderately severe cerebral arteriosclerosis	Yes
826	2	86	F	CONTROL	No AD, moderate CAA	Yes
854	2	96	F	CONTROL	Control, moderate cerebrovascular arteriosclerosis, Argyrophilic grain disease	Yes
870	2	90	F	CONTROL	Mild argyrophilic grain disease, moderate CAA, fine to use as control brain	Yes
1028	2	89	F	CONTROL	Nil of note	Yes
1082	2	94	M	CONTROL	Mild/early stage Lewy body pathology, TDP43 pathology associated with ageing	Yes
1089	2	78	F	CONTROL	No significant abnormalities	No
1092	2	86	F	CONTROL	Normal brain, moderate CAA	Yes
714	3	73	M	CONTROL	NORMAL	No
749	3	92	F	AD	AD possible, Argyrophilic grain disease, CVD	Yes
818	3	87	F	CONTROL	Apart from mild small vessel disease, no abnormalities seen	Yes
845	3	92	F	AD	Moderately severe atherosclerotic cerebrovascular disease, Argyrophilic grain disease	Yes
918	3	85	F	CONTROL	Control, microinfarct in hippocampus	Yes
929	3	81	M	CONTROL	Fine to use as control. Argyrophilic grain disease	
945	3	80	M	CONTROL	Probable argyrophilic grain disease, Mild AD changes	Yes
977	3	91	F	CONTROL	Use as control but not for comparison with VaD. Moderate SVD	Yes

Case ID	Braak stage	Age	Gender	Clinical Diagnosis	Histological Diagnosis	Frozen tissue
1039	3	83	F	CONTROL	Minimal TDP43 pathology in amygdala (fine to use as control)	Yes
1096	3	94	M	AD	DLB, AD probable	Yes
753	4	97	M	AD	Severe CAA, AD possible (mild AD-type changes)	Yes
760	4	95	M	AD / VAD	AD probable, mod CAA, mod small vessel disease	Yes
765	4	80	M	AD	AD probable, moderate CAA	No
794	4	80	F	AD	AD probable, moderate CAA	Yes
800	4	92	F	CONTROL	Braak tangle stage IV but does not fulfil CERAD criteria for AD	Yes
856	4	81	M	AD	AD probable, severe capillary CAA	Yes
898	4	88	M	DEMENTIA	AD probable, moderately severe atheromatous large vessel disease and arteriosclerotic small vessel disease, severe CAA	Yes
955	4	87	F	AD	AD definite, moderate SVD	Yes
992	4	80	M	VaD	According to NIA-AA guidelines, there is a high likelihood that this patients dementia was caused by Alzheimer's pathology	Yes
1076	4	86	M	AD	AD definite, AD-associated TDP43 pathology, hippocampal sclerosis	Yes
718	5	98	F	AD	AD probable, mod severe	No
723	5	84	F	AD	AD definite, very mild LBD	No
758	5	86	M	AD / VAD	AD probable, moderately severe CAA	Yes
763	5	80	F	AD	AD definite	Yes
809	5	83	M	VAD	AD probable	Yes
816	5	84	M	DEMENTIA	AD definite, limbic LBD, moderate small vessel disease	Yes
833	5	73	F	MILD AD	AD definite, moderate CAA	Yes
839	5	75	M	AD	AD definite	Yes
696	6	78	M	AD / DLB	AD	No
706	6	76	F	LBD / AD	AD, LBD	No
717	6	85	M	AD	AD probable. Mod sev sma ves dis	No
737	6	67	F	AD	AD definite, moderate CAA, small vessel disease	Yes
742	6	87	F	AD	AD definite, DLB (moderate cortical involvement)	Yes
745	6	84	F	AD	AD definite, severe arteriosclerosis, mod CAA	Yes
773	6	69	M	VAD	AD severe	Yes
864	6	86	F	AD	AD definite (severe)	Yes
882	6	78	F	SENILE DEMENTIA	AD definite	Yes
885	6	88	F	AD	AD definite	Yes
1075	6	79	M	AD	AD definite, mild DLB	Yes
1003	6	69	M	AD	AD definite	Yes

Appendix F Removal of pigmented neuromelanin

On examination of the first immunostaining for 4G8 and AT8, I realised that it was difficult to evaluate the amount of immunostaining of the investigated protein in the LC because the brown chromogen used (DAB) is of a similar colour to, and therefore difficult to distinguish from, the neuromelanin pigment of the LC cells (Figure 64).

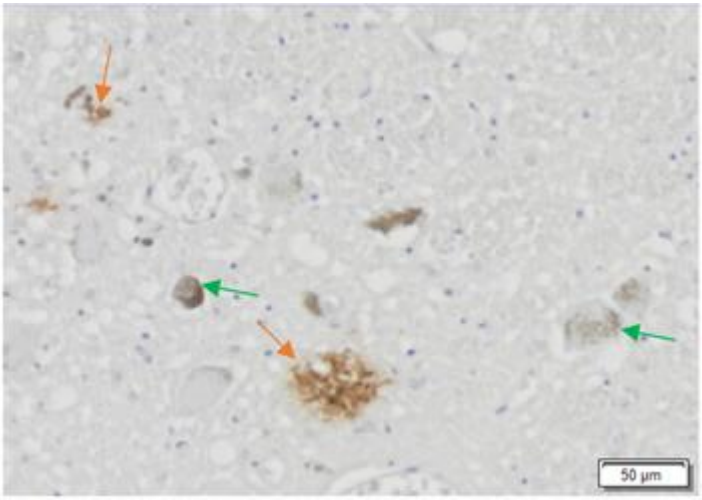


Figure 64. Image of unbleached LC section stained with 4G8

The NM within LC cells (green arrows) has a similar hue to the staining of the amyloid plaques (red arrows) and ImageJ is unable to separate amyloid staining with NM pigment left untreated.

Bleaching the NM prior to performing IHC appears to be the most effective way to enable accurate evaluation of protein load [337,338]. Typically this is done either by using potassium permanganate or hydrogen peroxide. Using potassium permanganate has the advantage of being quicker (taking approximately 35 minutes) but is more likely to damage tissue and to affect the affinity of certain antibodies [339]. Whereas bleaching with hydrogen peroxide has less of a negative impact on the tissue but takes much longer, with protocols ranging from 18hrs [340] to 48hrs [341]. When optimising the method for bleaching with hydrogen peroxide treatment with 10% hydrogen peroxide for 16 hours was not sufficient to bleach the NM pigment whereas bleaching for 21 hours successfully removed the NM (Figure 65). Some authors suggest it is possible to reduce the time taken to bleach with hydrogen peroxide to 40 minutes by using a water bath at 65° [328,342]. However when this method was trialled, 40 minutes was not sufficient to bleach NM (Figure 66).

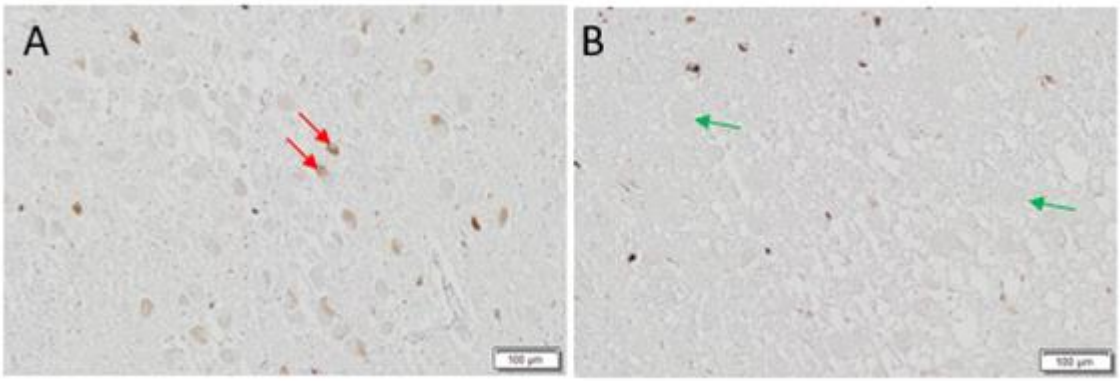


Figure 65. Comparison of case 971 stained with 4G8 bleached with 10% H_2O_2 for 16 hours (A) or 21 hours (B)

After 16 hours NM pigment is still visible (red arrows) whereas after 21 hours the NM pigment is fully bleached and the LC cell outline can only just be seen (green arrows).

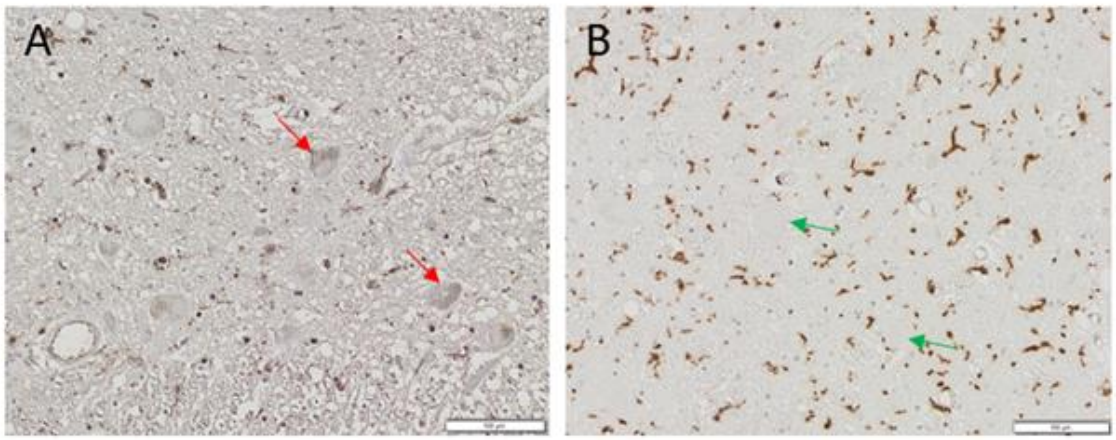


Figure 66. Comparison of case 885 stained with IBA1 bleached with H_2O_2 for 40minutes in a water bath (A) or overnight at room temperature (B)

Using the water bath for 40 minutes was not sufficient to bleach all of the neuromelanin.

Alternatively another option was to add nickel to DAB, which enhanced the staining to a brown-black colour and may better distinguish from the brown NM. However when trialled the staining was not dark enough to be separated from the NM which when heavily deposited is also a deep brown colour (Figure 67).

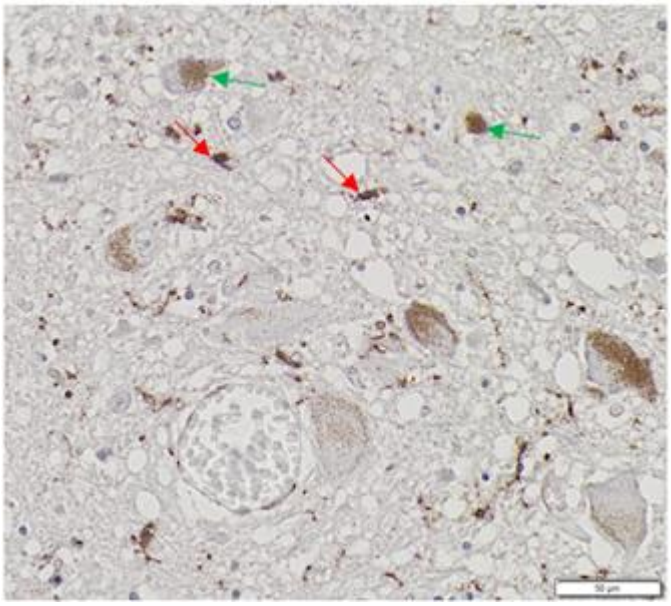


Figure 67. Case 1039 stained with IBA1 using DAB with Nickel

Unfortunately the nickel staining (red arrows) did not make the protein staining dark enough to distinguish it from the NM pigment (green arrows)

Unexpectedly bleaching of the slides before IHC not only bleached the NM pigment from the LC cells but actually enhanced the immunostaining by the antibody for 4G8 and AT8. This has been shown in other studies e.g. when NM was bleached in the SN 2.5 times more NFTs were observed than when the staining was completed without bleach [337]. In order to draw comparisons within cases between protein load in the LC and temporal cortex both sections were pre-treated with hydrogen peroxide for the same amount of time.

Appendix G Homogenisation of brain tissue

1) Make up Lysis buffer: Add 7ml of RIPA buffer (Thermo Scientific; 89900), one protease inhibitor tablet (Sigma, Roche; complete Mini, 046931240010), and one phosphatase inhibitor tablet (Thermo Scientific; Pierce^{TM}; 88667) to a 15 ml falcon tube, and vortex until completely dissolved. Keep on wet ice.

2) Add 500µl of lysis buffer to 100mg of each tissue sample in a 2ml screw-cap tube. Homogenise for ~40 seconds until no pieces of tissue remained.

3) Centrifuge homogenised samples at 13,000 rpm for 15 minutes at 4°C.

4) Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in labelled 1.5ml tubes kept on ice; discard the pellet.

5) Aliquot 10µl for the BCA Assay, 100µl for the MSD assay. Snap-freeze on dry ice and store at -80^{o}C.

Appendix H Determination of protein concentration

Use BCA protein assay kit (Thermo Scientific, Pierce™; 23227).

1) Prepare standards (A to I) as specified in the manual of BCA assay kit. Use one 2mg/ml ampulla from the BCA assay kit as the stock solution. For the diluent use the same lysis buffer as used for the homogenised samples. Prepare 9 standards:
    - A (2000ug/ul), B (1500ug/ul), C (1000ug/ul), D (750ug/ul), E (500ug/ul), F (250ug/ul), G (125ug/ul), H (25ug/ul), I (0ug/ul)

2) Prepare the BCA working reagent by mixing 50 parts of BCA reagent A with 1 part BCA reagent B.

3) Dilute the samples tenfold by mixing 27µl lysis buffer and 3µl sample into 0.5 ml Eppendorfs.

4) Vortex all diluted samples and standards. Then pipette 10µl of each into the wells of a Greiner 96 flat bottom polystyrene well plate (Sigma; M3061), in triplicates for both sample and standard. Use a new pipette tip for each well to maximise precision and touch the pipette to the sides of the wells when unloading the sample.

5) Add 200µl of working reagent to each well, gently mix, cover the plate with foil, and place it in a 37°C incubator for 30 minutes.

6) Load the sample plate in an Infinite F200 PRO plate reader, and use iControl1.9 software (Tecan; Austria GmbH)

7) Select BCA, choose the appropriate wells and select the appropriate plate (Gre96Fl: Greiner 96 flat transparent) and wavelength (570 nm)

An Excel spreadsheet with absorbance values for each well is produced, from which a standard curve can be plotted

Appendix I Results tables from MesoScale Discovery Assay

Median values and interquartile ranges for each Braak group are shown in the tables below for each of the 30 markers analysed in each brain area along with significance values from Kruskal-Wallis tests.

Table 18. Average protein concentrations in the LC of the 10 markers on the Chemokine plate by Braak stage group

Marker	Braak 0-II (*n*=19)	Braak III-IV (*n*=18)	Braak V-VI (*n*=15)	*p* value	*p* value, adjusted for age and gender
Eotaxin	$1.7x10^{-7}$ [$1.2x10^{-7}$ – $3.3x10^{-7}$]	$1.5x10^{-7}$ [$0.7x10^{-7}$ – $2.8x10^{-7}$]	$0.9x10^{-7}$ [$0.7x10^{-7}$ – $2.4x10^{-7}$]	0.150	
Eotaxin-3	$3.4x10^{-7}$ [$2.8x10^{-7}$ 4.5– $x10^{-7}$]	$4.4x10^{-7}$ [$2.7x10^{-7}$ – $5.9x10^{-7}$]	$3.2x10^{-7}$ [$1.5x10^{-7}$ – $6.4x10^{-7}$]	0.781	
IL-8 (HA)	**$2.4x10^{-6}$ [$1.5x10^{-6}$ – $3.5x10^{-6}$]**	**$2.6x10^{-6}$ [$0.9x10^{-6}$ – $6.6x10^{-6}$]**	**$1.5x10^{-6}$ [$0.4x10^{-6}$ – $2.3x10^{-6}$]**	**0.046**	**0.044**
IP-10	$1.9x10^{-7}$ [$0.8x10^{-7}$ – $2.5x10^{-7}$]	$1.5x10^{-7}$ [$1.0x10^{-7}$ – $4.2x10^{-7}$]	$2.0x10^{-7}$ [$1.2x10^{-7}$ – $3.3x10^{-7}$]	0.653	
MCP-1	$6.9x10^{-7}$ [$3.8x10^{-7}$ – $10.9x10^{-7}$]	$6.8x10^{-7}$ [$4.2x10^{-7}$ – $22.6x10^{-7}$]	$8.5x10^{-7}$ [$5.9x10^{-7}$ – $12.4x10^{-7}$]	0.519	
MCP-4	$3.2x10^{-7}$ [$2.4x10^{-7}$ – $4.0x10^{-7}$]	$2.7x10^{-7}$ [$2.2x10^{-7}$ – $3.7x10^{-7}$]	$2.1x10^{-7}$ [$1.8x10^{-7}$ – $2.8x10^{-7}$]	0.038	
TARC	**$2.7x10^{-8}$ [$1.1x10^{-8}$ – $8.3x10^{-8}$]**	**$3.2x10^{-8}$ [$0.8x10^{-8}$ – $5.3x10^{-8}$]**	**$1.0x10^{-8}$ [$0.3x10^{-8}$ – $2.1x10^{-8}$]**	**0.026**	**0.028**
MDC	**$3.5x10^{-7}$ [$2.5x10^{-7}$ – $8.1x10^{-7}$]**	**$2.9x10^{-7}$ [$1.3x10^{-7}$ – $4.6x10^{-7}$]**	**$1.8x10^{-7}$ [$1.2x10^{-7}$ – $3.7x10^{-7}$]**	**0.027**	**0.002***
MIP-1β	**$2.5x10^{-7}$ [$2.0x10^{-7}$– $3.6x10^{-7}$]**	**$2.6x10^{-7}$ [$1.5x10^{-7}$ – $3.7x10^{-7}$]**	**$1.5x10^{-7}$ [$1.0x10^{-7}$ – $2.1x10^{-7}$]**	**0.006**	**0.05**
MIP-1α	$2.5x10^{-7}$ [$1.8x10^{-7}$ – $3.3x10^{-7}$]	$2.6x10^{-7}$ [$1.3x10^{-7}$ – $4.9x10^{-7}$]	$1.6\ x10^{-7}$ [$0.7x10^{-7}$ – $3.3x10^{-7}$]	0.225	

*significant *p* values *p*<0.005 (Bonferroni corrected for multiple tests).

***p*<0.05 shown in bold**

p values calculated using Kruskal-Wallis test

Data presented as median [Interquartile range]

Table 19. Average protein concentrations in the temporal cortex of the 10 markers on the Chemokine plate by Braak stage group

Marker	Braak 0-II (*n*=19)	Braak III-IV (*n*=18)	Braak V-VI (*n*=15)	*p* value
Eotaxin	$1.0x10^{-7}$ [$0.6x10^{-7}$ – $1.7x10^{-7}$]	$1.1x10^{-7}$ [$0.7x10^{-7}$ – $1.5x10^{-7}$]	$1.1x10^{-7}$ [$0.8x10^{-7}$ – $2.5x10^{-7}$]	0.510
Eotaxin-3	$2.1x10^{-7}$ [$1.5x10^{-7}$ – $3.6x10^{-7}$]	$5.1x10^{-7}$ [$2.1x10^{-7}$ – $7.8x10^{-7}$]	$3.2x10^{-7}$ [$1.5x10^{-7}$ – $5.5x10^{-7}$]	0.105
IL-8 (HA)	$1.5x10^{-6}$ [$1.1x10^{-6}$ – $2.9x10^{-6}$]	$1.9x10^{-6}$ [$0.8x10^{-6}$ – $2.9x10^{-6}$]	$1.7x10^{-6}$ [$1.1x10^{-6}$ – $4.8x10^{-6}$]	0.924
IP-10	$2.7x10^{-7}$ [$1.7x10^{-7}$ – $4.3x10^{-7}$]	$4.3x10^{-7}$ [$2.3x10^{-7}$ – $12.3x10^{-7}$]	$4.7x10^{-7}$ [$3.0x10^{-7}$ – $7.3x10^{-7}$]	0.314
MCP-1	$6.0x10^{-7}$ [$3.3x10^{-7}$ – $10.3x10^{-7}$]	$7.3x10^{-7}$ [$4.4x10^{-7}$ – $14.1x10^{-7}$]	$11.3x10^{-7}$ [$7.3x10^{-7}$ – $16.3x10^{-7}$]	0.323
MCP-4	$2.0x10^{-7}$ [$1.6x10^{-7}$ – $2.5x10^{-7}$]	$1.7x10^{-7}$ [$1.6x10^{-7}$ – $2.5x10^{-7}$]	$2.4x10^{-7}$ [$1.6x10^{-7}$ – $2.5x10^{-7}$]	0.084
TARC	$2.2x10^{-8}$ [$0.9x10^{-8}$ – $3.6x10^{-8}$]	$2.9x10^{-8}$ [$2.1x10^{-8}$ – $4.7x10^{-8}$]	$2.4x10^{-8}$ [$1.2x10^{-8}$ – $4.1x10^{-8}$]	0.393
MDC	$2.7x10^{-7}$ [$1.9x10^{-7}$ – $3.6x10^{-7}$]	$2.7\ x10^{-7}$ [$1.4x10^{-7}$ – $3.8x10^{-7}$]	$3.3\ x10^{-7}$ [$2.7x10^{-7}$ – $8.9x10^{-7}$]	0.174
MIP-1β	$1.9\ x10^{-7}$ [$1.7x10^{-7}$ – $2.3x10^{-7}$]	$2.6\ x10^{-7}$ [$1.6x10^{-7}$ – $4.2x10^{-7}$]	$1.8\ x10^{-7}$ [$1.7x10^{-7}$ – $3.2x10^{-7}$]	0.242
MIP-1α	$1.3x10^{-7}$ [$1.0x10^{-7}$ – $1.6x10^{-7}$]	$1.7x10^{-7}$ [$1.0x10^{-7}$ – $3.0x10^{-7}$]	$1.5x10^{-7}$ [$1.3x10^{-7}$ – $2.9x10^{-7}$]	0.235

*significant *p* values $p<0.005$ (Bonferroni corrected for multiple tests).
p values calculated using Kruskal-Wallis test
Data presented as median [Interquartile range]

Table 20. Average protein concentrations in the LC of the 10 markers on the Proinflammatory plate by Braak stage group

Marker	Braak 0-II (*n*=19)	Braak III-IV (*n*=18)	Braak V-VI (*n*=15)	*p* value
IFN-γ	$0.6x10^{-8}$ [$0.4x10^{-8}$ – $1.0x10^{-8}$]	$0.7\ x10^{-8}$ [$0.4x10^{-8}$ – $0.9x10^{-8}$]	$0.7\ x10^{-8}$ [$0.5x10^{-8}$ – $0.9x10^{-8}$]	0.928
IL-β	$0.8\ x10^{-8}$ [$0.5x10^{-8}$ – $4.6x10^{-8}$]	$1.5\ x10^{-8}$ [$0.7x10^{-8}$ – $3.9x10^{-8}$]	$0.9\ x10^{-8}$ [$0.4x10^{-8}$ – $3.1x10^{-8}$]	0.313
IL-10	$0.1x10^{-8}$ [$0.09x10^{-8}$ – $0.2x10^{-8}$]	$0.1x10^{-8}$ [$0.1x10^{-8}$ – $0.2x10^{-8}$]	$0.1\ x10^{-8}$ [$0.09x10^{-8}$ – $0.1x10^{-8}$]	0.124
IL-12p70	$0.5x10^{-8}$ [$0.3x10^{-8}$ – $0.7x10^{-8}$]	$0.4\ x10^{-8}$ [$0.3x10^{-8}$ – $0.8x10^{-8}$]	$0.4\ x10^{-8}$[$0.3x10^{-8}$ – $0.6x10^{-8}$]	0.766
IL-13	$7.3x10^{-8}$ [$5.4x10^{-8}$ – $8.5x10^{-8}$]	$7.5\ x10^{-8}$ [$5.5x10^{-8}$ – $9.1x10^{-8}$]	$6.4\ x10^{-8}$ [$5.4x10^{-8}$ – $7.7x10^{-8}$]	0.599
IL-2	$0.3\ x10^{-8}$ [$0.2x10^{-8}$ – $0.4x10^{-8}$]	$0.3\ x10^{-8}$ [$0.3x10^{-8}$ – $0.5x10^{-8}$]	$0.3\ x10^{-8}$ [$0.2x10^{-8}$ – $0.4x10^{-8}$]	0.160
IL-4	$0.3\ x10^{-8}$ [$0.2x10^{-8}$ – $0.3x10^{-8}$]	$0.3\ x10^{-8}$ [$0.2x10^{-8}$ – $0.3x10^{-8}$]	$0.2\ x10^{-8}$ [$0.2x10^{-8}$ – $0.3x10^{-8}$]	0.121
IL-6	$3.7\ x10^{-8}$ [$1.7x10^{-8}$ – $8.7x10^{-8}$]	$8.1\ x10^{-8}$ [$2.6x10^{-8}$ – $33.7x10^{-8}$]	$5.3\ x10^{-8}$ [$2.7x10^{-8}$ – $10.8x10^{-8}$]	0.412
IL-8	$1.8x10^{-7}$[$0.8x10^{-7}$ – $3.3x10^{-7}$]	$2.2\ x10^{-7}$ [$1.4x10^{-7}$ – $4.3x10^{-7}$]	$1.9\ x10^{-7}$ [$1.4x10^{-7}$ – $3.2x10^{-7}$]	0.792
TNFα	$0.4\ x10^{-8}$ [$0.4x10^{-8}$ – $0.7x10^{-8}$]	$0.4\ x10^{-8}$ [$0.4x10^{-8}$ – $0.5x10^{-8}$]	$0.4\ x10^{-8}$ [$0.3x10^{-8}$ – $0.6x10^{-8}$]	0.594

*significant *p* values $p<0.005$ (Bonferroni corrected for multiple tests).
p values calculated using Kruskal-Wallis test
Data presented as median [Interquartile range]

Table 21. Average protein concentrations in the temporal cortex of the 10 markers on the Proinflammatory plate by Braak stage group

Marker	Braak 0-II (*n*=19)	Braak III-IV (*n*=18)	Braak V-VI (*n*=15)	*p* value
IFN-γ	$0.8x10^{-8}$ [$0.5x10^{-8}$ – $1.1x10^{-8}$]	$0.8\ x10^{-8}$ [$0.6x10^{-8}$ – $1.3x10^{-8}$]	$2.4x10^{-8}$ [$0.5x10^{-8}$ – $0.9x10^{-8}$]	0.241
IL-β	$2.6\ x10^{-8}$ [$1.6x10^{-8}$ – $6.0x10^{-8}$]	$3.1x10^{-8}$ [$2.3x10^{-8}$ – $4.0x10^{-8}$]	$0.9\ x10^{-8}$ [$0.4x10^{-8}$ – $3.1x10^{-8}$]	0.592
IL-10	$0.1x10^{-8}$ [$0.09x10^{-8}$ – $0.2x10^{-8}$]	$0.2x10^{-8}$ [$0.1x10^{-8}$ – $0.2x10^{-8}$]	$0.1\ x10^{-8}$ [$0.08x10^{-8}$ – $0.2x10^{-8}$]	0.129
IL-12p70	$0.5x10^{-8}$ [$0.4x10^{-8}$ – $0.6x10^{-8}$]	$0.4\ x10^{-8}$ [$0.3x10^{-8}$ – $0.6x10^{-8}$]	$0.4\ x10^{-8}$[$0.3x10^{-8}$ – $0.5x10^{-8}$]	0.847
IL-13	$4.7x10^{-8}$ [$3.7x10^{-8}$ – $5.9x10^{-8}$]	$5.0x10^{-8}$ [$4.0x10^{-8}$ – $5.6x10^{-8}$]	$5.2\ x10^{-8}$ [$4.2x10^{-8}$ – $6.1x10^{-8}$]	0.885
IL-2	$0.3\ x10^{-8}$ [$0.2x10^{-8}$ – $0.4x10^{-8}$]	$0.3\ x10^{-8}$ [$0.3x10^{-8}$ – $0.4x10^{-8}$]	$0.2x10^{-8}$ [$0.2x10^{-8}$ – $0.4x10^{-8}$]	0.140
IL-4	$0.2x10^{-8}$ [$0.2x10^{-8}$ – $0.3x10^{-8}$]	$0.2x10^{-8}$ [$0.2x10^{-8}$ – $0.3x10^{-8}$]	$0.2x10^{-8}$ [$0.2x10^{-8}$ – $0.3x10^{-8}$]	0.628
IL-6	$5.1x10^{-8}$ [$1.9x10^{-8}$ –$19.9x10^{-8}$]	$8.3x10^{-8}$ [$3.6x10^{-8}$ – $17.4x10^{-8}$]	$5.0x10^{-8}$ [$2.7x10^{-8}$ – $14.7x10^{-8}$]	0.708
IL-8	$2.6x10^{-7}$[$1.6x10^{-7}$ – $7.0x10^{-7}$]	$2.9x10^{-7}$ [$1.8x10^{-7}$ – $8.6x10^{-7}$]	$2.3x10^{-7}$ [$1.5x10^{-7}$ – $5.7x10^{-7}$]	0.826
TNFα	$0.6x10^{-8}$ [$0.5x10^{-8}$ – $0.7x10^{-8}$]	$0.6x10^{-8}$ [$0.4x10^{-8}$ – $0.8x10^{-8}$]	$0.5x10^{-8}$ [$0.4x10^{-8}$ – $0.7x10^{-8}$]	0.533

*significant *p* values $p<0.005$ (Bonferroni corrected for multiple tests).
p values calculated using Kruskal-Wallis test
Data presented as median [Interquartile range]

Table 22. Average protein concentrations in the LC of the 10 markers on the Cytokine plate by Braak stage group

Marker	Braak 0-II (*n*=19)	Braak III-IV (*n*=18)	Braak V-VI (*n*=15)	*p* value	*p* value, adjusted for age and gender
IL-α	$4.2x10^{-8}$ [$2.2x10^{-8}$ – $11.6x10^{-8}$]	$4.0x10^{-8}$ [$2.3x10^{-8}$ – $7.7x10^{-8}$]	$2.9x10^{-8}$ [$1.8x10^{-8}$ – $7.7x10^{-8}$]	0.403	
IL-15	$8.0x10^{-8}$ [$6.4x10^{-8}$ – $9.6x10^{-8}$]	$9.9x10^{-8}$ [$6.4x10^{-8}$ – $13.5x10^{-8}$]	$9.4x10^{-8}$ [$7.3x10^{-8}$ – $12.0x10^{-8}$]	0.251	
IL-12/IL-23p70	$1.2x10^{-8}$ [$1.0x10^{-8}$ – $1.7x10^{-8}$]	$1.3x10^{-8}$ [$1.0x10^{-8}$ – $1.8x10^{-8}$]	$1.4x10^{-8}$ [$1.3x10^{-8}$ – $1.7x10^{-8}$]	0.480	
IL-16	$5.8x10^{-6}$ [$3.3x10^{-6}$ – $8.9x10^{-6}$]	$6.7x10^{-6}$ [$5.0x10^{-6}$ – $12.5x10^{-6}$]	$8.6x10^{-6}$ [$7.0x10^{-6}$ – $10.4x10^{-6}$]	0.101	
IL-17A	$2.5x10^{-8}$ [$1.6x10^{-8}$ – $3.0x10^{-8}$]	$1.8x10^{-8}$ [$1.5x10^{-8}$ – $2.5x10^{-8}$]	$1.7x10^{-8}$ [$1.7x10^{-8}$ – $2.0x10^{-8}$]	0.037	0.066
GM-CSF	$0.1x10^{-8}$ [$0.08x10^{-8}$ – $0.2x10^{-8}$]	$0.2x10^{-8}$ [$0.1x10^{-8}$ – $0.2x10^{-8}$]	$0.09x10^{-8}$ [$0.07x10^{-8}$ – $0.1x10^{-8}$]	0.038	0.066
IL-5	$0.1x10^{-8}$ [$0.06x10^{-8}$ – $0.2x10^{-8}$]	$0.2x10^{-8}$ [$0.09x10^{-8}$ – $0.02x10^{-8}$]	$0.1x10^{-8}$ [$0.06x10^{-8}$ – $0.2x10^{-8}$]	0.132	
IL-7	$0.3x10^{-8}$ [$0.3x10^{-8}$ – $0.4x10^{-8}$]	$0.4x10^{-8}$ [$0.2x10^{-8}$ – $0.6x10^{-8}$]	$0.4x10^{-8}$ [$0.2x10^{-8}$ – $0.4x10^{-8}$]	0.535	
TNFβ	$0.1x10^{-8}$ [$0.09x10^{-8}$ – $1.8x10^{-8}$]	$0.1x10^{-8}$ [$0.07x10^{-8}$ – $0.2x10^{-8}$]	$0.1x10^{-8}$ [$0.08x10^{-8}$ – $0.2x10^{-8}$]	0.727	
VEGF	$9.9x10^{-8}$ [$3.8x10^{-8}$ – $21.6x10^{-8}$]	$7.4x10^{-8}$ [$4.3x10^{-8}$ – $22.2x10^{-8}$]	$9.5x10^{-8}$ [$3.1x10^{-8}$ – $22.0x10^{-8}$]	0.994	

*significant *p* values *p*<0.005 (Bonferroni corrected for multiple tests).
p values calculated using Kruskal-Wallis test
Data presented as median [Interquartile range]

Table 23. Average protein concentrations in the temporal cortex of the 10 markers on the Cytokine plate by Braak stage group

Marker	Braak 0-II (*n*=19)	Braak III-IV (*n*=18)	Braak V-VI (*n*=15)	*p* value	*p* value, adjusted for age and gender
IL-α	$4.0x10^{-8}$ [$2.8x10^{-8}$ – $6.2x10^{-8}$]	$5.7x10^{-8}$ [$3.9x10^{-8}$ – $8.4x10^{-8}$]	$3.0x10^{-8}$ [$1.9x10^{-8}$ – $11.8x10^{-8}$]	0.424	
IL-15	**$6.7x10^{-8}$ [$6.0x10^{-8}$ – $8.1x10^{-8}$]**	**$7.5x10^{-8}$ [$6.4x10^{-8}$ – $9.5x10^{-8}$]**	**$9.3x10^{-8}$ [$7.6x10^{-8}$ – $10.7x10^{-8}$]**	**0.024**	**0.006**
IL-12/IL-23p70	$1.3x10^{-8}$ [$1.1x10^{-8}$ – $1.9x10^{-8}$]	$1.6x10^{-8}$ [$1.1x10^{-8}$ – $2.0x10^{-8}$]	$1.2x10^{-8}$ [$1.0x10^{-8}$ – $1.8x10^{-8}$]	0.455	
IL-16	$4.2x10^{-6}$ [$3.9x10^{-6}$ – $6.9x10^{-6}$]	$6.0x10^{-6}$ [$3.7x10^{-6}$ – $8.4x10^{-6}$]	$6.3x10^{-6}$ [$4.3x10^{-6}$ – $7.7x10^{-6}$]	0.586	
IL-17A	$1.3x10^{-8}$ [$1.1x10^{-8}$ – $1.7x10^{-8}$]	$1.5x10^{-8}$ [$0.9x10^{-8}$ – $1.8x10^{-8}$]	$1.7x10^{-8}$ [$1.3x10^{-8}$ – $1.9x10^{-8}$]	0.192	
GM-CSF	$0.1x10^{-8}$ [$0.1x10^{-8}$ – $0.2x10^{-8}$]	$0.2x10^{-8}$ [$0.1x10^{-8}$ – $0.3x10^{-8}$]	$0.1x10^{-8}$ [$0.07x10^{-8}$ – $0.2x10^{-8}$]	0.254	
IL-5	$0.08x10^{-8}$ [$0.05x10^{-8}$ – $0.2x10^{-8}$]	$0.2x10^{-8}$ [$0.09x10^{-8}$ – $0.2x10^{-8}$]	$0.09x10^{-8}$ [$0.05x10^{-8}$ – $0.2x10^{-8}$]	0.188	
IL-7	$0.4x10^{-8}$ [$0.3x10^{-8}$ – $0.6x10^{-8}$]	$0.6x10^{-8}$ [$0.4x10^{-8}$ –$0.8\ x10^{-8}$]	$0.4x10^{-8}$ [$0.2x10^{-8}$ – $0.5x10^{-8}$]	0.070	
TNFβ	$0.1x10^{-8}$ [$0.09x10^{-8}$ – $0.2x10^{-8}$]	$0.1x10^{-8}$ [$0.1x10^{-8}$ – $0.2x10^{-8}$]	$0.1x10^{-8}$ [$0.1x10^{-8}$ – $0.2x10^{-8}$]	0.916	
VEGF	$2.0x10^{-8}$ [$1.0x10^{-8}$ – $3.7x10^{-8}$]	$2.5x10^{-8}$ [$1.2x10^{-8}$ – $4.9x10^{-8}$]	$2.2x10^{-8}$ [$0.7x10^{-8}$ – $4.1x10^{-8}$]	0.797	

*significant *p* values *p*<0.005 (Bonferroni corrected for multiple tests).
***p*<0.05 shown in bold**
p values calculated using Kruskal-Wallis test
Data presented as median [Interquartile range]

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