Shedding light on the molecular mechanisms that underlie the clinical heterogeneity of different synucleinopathies.

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Abstract:

Neurodegenerative diseases such as Parkinson's disease (PD) don't fall directly into the category of dementia. However, diseases such as dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD) are classified as types of dementia. Despite this overlap there is significant clinical heterogeneity between the subtypes of these diseases, as well as their newly classified cousins. Collectively we can refer to these diseases as synucleinopathies, as they involve the aggregation of α-synuclein in Lewy bodies and neurites. These diseases are clinically heterogenous; how can neurologists and psychiatrists differentiate between these diseases in the clinic? In order to be able to design treatments for patients which are effective, we have to understand the molecular mechanisms that are at play and how they differ. This will eventually also reduce the overdiagnosis and consequent underdiagnosis of these diseases. The aim of this project is to discern how the presence of molecular markers differs in the brains of deceased patients who have different synucleinopathies, and further how other factors such as duration of the disease and sex affect the presence of these markers. Neuropathological analysis was required for the confirmation of diagnosis, and from these carefully selected cases we can carry out analysis on the donated brain tissue to ascertain the causative factors. Here we stained brain sections of the cingulate cortex from the brains of patients who had PD, PDD and DLB. They were stained for a number of markers that are implicated in synucleinopathies, including tau, α-synuclein, β-amyloid and human leukocyte antigen (HLA). The stained tissue was analysed by high-throughput imaging to quantify the presence of these markers in the grey matter. HLA can be used as a marker of immunological activity in tissue as it plays a key role in self-protein processing, and it is a marker of microglial presence. From the area quantification analysis, we can conclude that there was a higher presence of HLA in cases with DLB in comparison to those with PD. Correlating this finding and other trends we identified with genetic and transcriptomic profile studies can help us elucidate new mechanisms that underlie the heterogenous clinical profiles of synucleinopathies.

Introduction:

Neurodegenerative Diseases, Dementia and Synucleinopathies:

Neurodegenerative diseases are associated with the presence of abnormally folded proteins that persist in the tissue due to failures in their clearance. For example, with amyloid there is a misfolding of normal host protein from alpha-rich to beta-rich (resulting in β-amyloid formation, a common occurrence in disease such as Alzheimer's Disease (AD)). Proteins misfold for a number of reasons: infectious, where there is a propagated conformational change such as in variant Creutzfeldt Jakob Disease[1]; genetic, where there is an inherited propensity for a protein to misfold such as in fatal familial insomnia[2] (a prion disease of the brain that is almost always caused by a mutation to the protein PrP^{C}[3]); and also as a consequence of ageing. In the latter we see a failure of normal clearance mechanisms, due to the energy-requiring nature of these processes[4].

The word 'dementia' has wide-ranging biopsychosocial definitions. It is an under-recognized cause of long-term morbidity and mortality. However, despite the arduous efforts over the past half-century, these conditions are still incredibly elusive. These are diseases of ageing, and thus their demographic effects are large. The burden of these chronic degenerative diseases is increasing rapidly, but it is important to consider whether this is due to improved diagnosis or increases in age-specific prevalence. In the UK, mortality due to chronic degenerative diseases is partly due to an ageing population[5].

Parkinson's disease (PD) affects 1% of people over the age of 60[6], and is characterised by bradykinesia, akinesia and rigidity as well as tremors of the hands and jaw and the typical shuffling (and fenestrated) gait. This neurodegenerative disease was first described by James Parkinson in 1817 as "the shaking palsy". Symptoms emerge usually in the 5th or 6^{th} decade (in the sporadic form). Patients also experience non-motor symptoms[7], such as with sleep (in the form of rapid eye movement sleep behaviour disorder (RBD)), olfaction, bowels, and cognitive deficits, decreased motivation, depression, and lack of affection. PD is characterized by a loss of dopaminergic cells in the substantia nigra pars compacta (SN_{pc}) that project in the nigrostriatal pathway, and the consequences of this damage reflect the role of this area of the brain (the basal ganglia) in procedural learning[8]. The destruction of SN_{pc} neurons is often preceded by the appearance of Lewy bodies, which mainly consist of aggregates of insoluble intraneural components of α-synuclein and ubiquitin[9]. The genetic component of the disease is correlated with these insoluble aggregates[10]. A defect in chromosome 4 seems to lead to duplication of the gene coding for α-synuclein[11]. More common than this mutation however is the parkin gene on chromosome 6, or the more recently elucidated LRRK2 mutation[12].

Dementia with Lewy bodies (DLB) is, like PD, a disease where Lewy bodies and Lewy neurites are formed from the aggregation of α-synuclein[13]. The difference between this condition and PD is that we see features of cognitive decline resembling a dementia phenotype superimposed on top of the motor symptoms of PD. Interestingly, post-mortem findings with retrospective history analysis found that a cohort with

considerable Lewy body (LB) pathology had a 50/50 split in their clinical presentation[14]. Half of the cases had a dementia phenotype that was considerably different to other subtypes, whilst the other half a generalised overall cognitive decline with very little discernible DLB pathology. Consequently, we can appreciate the clinical heterogeneity and the challenges it poses to diagnosis and treatment.

When cognitive impairment accompanies Parkinsonism, the challenges for the patient are heightened. PDD is an example of the more extreme end of the scale when it comes to the cognitive decline in patients with PD. Clinical heterogeneity is highlighted here again, with some patients having mild cognitive impairment and being able to manage their daily lives, contrasted with those with full-blown PDD, where daily functioning is impaired[15]. This cognitive impairment is also hard to pin down, as it varies in its severity, the areas of cognition it affects and the rate at which it changes during the progression of the disease[16].

Clinical heterogeneity and ambiguity:

The need for clearer distinction at the molecular as well as the clinical level is eloquently summarised in a recent review on DLB: “Although the end stage neuropathological findings in [DLB and PDD] may be similar, there can be little doubt that the clinical experience of the patients and their families will have been very different.”[17] Whilst DLB has dementia as a primary feature with motor features becoming relevant in the later stages of the diseases, the reverse is true for PDD, where Parkinsonian features are seen first, with a global cognitive decline experienced later.

Collectively, we can refer to these three diseases as synucleinopathies, as they are all associated with abnormal α-synuclein deposits[18]. These diseases have extensive clinical heterogeneity, and it is the goal of this research to help provide molecular bases for these differing pathways. As much as this is to inform future research into potential treatment, it is also important to provide patients with a pre-emptive explanation of what course their disease will take. This will inform both their treatment and prognosis.

Microglia in synucleinopathies:

Microglial cells are the macrophages of the central nervous system, and they act to regulate and maintain an immunological milieu within this region. Microglial cells scan their environment by moving their processes within a defined territory[19]. They can, similar to peripheral macrophages, be polarised to neuroprotective (M_2) or pro-inflammatory (M_1), and this distinction stems from the different receptors they present and the different effector mechanisms they carry out in response to binding to these upregulated receptors[20]. It is important to note however that this is an oversimplification and they actually show co-expression of both states[21], with some evidence of polarisation and thus this may be considered a slightly out-dated term. They exhibit heterogeneity in their expression patterns and are highly plastic[22]. There are a number of ways in which microglia could play a role: there could be too many ‘M_1’ microglia; not enough ‘M_2’; a switch from ‘M_2 to M_1’ (increasing

neuroinflammation); or microglial activation could itself be the initial insult. Microglia have been studied heavily, particularly with regards to their role in neurodegenerative diseases such as AD, where there is a tight interplay between neurovascular damage and microglial activation[22]. This neurovascular damage occurs at the blood-brain barrier (BBB) which forms a part of the neurovascular unit, regulating the entry of nutrients into, and clearance of toxic molecules out of, the brain tissue[23]. Examples of this include the aforementioned amyloid-β peptide in AD[24]. However, forms of PD, such as vascular parkinsonism, also exhibit this feature[25].

The microglial transcriptome has also revealed some interesting observations. PU.1 is a transcription factor that induces myeloid lineage commitment[26]. Studies on mice found that the disruption of this transcription factor led to a lack of microglia and significant haematopoietic abnormalities[27]. Mice with a PU.1 knockout exposed to wild-type microglia were found to have improved survivability, describing the importance of microglia in neuroprotection in diseases states such as familial amyotrophic lateral sclerosis[28]. Further to this, a genome-wide association study (GWAS) has recently shown that reduced levels of expression of transcription factors such as PU.1 are correlated with a delayed onset of AD[29]. Ultimately, one can see that studying the properties of microglia (and how they differ between different synucleinopathies) can shed insight into the potential causal mechanisms of neuroinflammation in these conditions.

Molecular markers in synucleinopathies:

Four different targets were used in this study to elucidate the molecular differences between the three different synucleinopathies. The first of these is α-synuclein. α-synuclein is a native protein in neurons, however its precise structure and function is still an issue of ongoing research[30]. Currently, α-synuclein is thought to promote synaptic adaptability in the form of vesicle budding, and thus trafficking at the synapse, via its role as a promoter of membrane curvature[31,32]. However, when deposited abnormally it forms Lewy bodies, and these are a key PD pathological finding. The presence of abnormal deposits would normally be cleared by microglia, however when they are deposited at such a high rate, they encourage a pro-inflammatory environment. The infiltration of T-cells specific for the exposed α-synuclein has been found at higher levels in PD tissue than healthy controls, signifying that central invasion of peripherally-derived lymphocytes can pave the way for autoimmunity to further the damage done in the brain tissue[33].

Even though a useful way of describing these diseases is as synucleinopathies, due to the involvement of AD-like symptoms (in the form of cognitive decline (dementia) as well as novel pathological findings), it is important to also study molecular markers that are associated with AD. One of these markers is hyperphosphorylated tau (P-tau). Tau, like α-synuclein, has a native role in the brain, and in this case, it is well defined[34]. It is involved in the stabilisation of microtubules as well as trafficking along the length of the axon. This form of tau is not folded and is highly soluble. In the disease state it undergoes hyperphosphorylation, and the protein takes on a misfolded and insoluble state – P-tau[35]. It folds and arranges into neurofibrillary tangles (NFTs) which are present in conditions known as tauopathies, of which AD is a prime example. PD was

not always thought to be a tauopathy, however a GWAS discovered new loci that increase the risk of PD, and one of them was microtubule-associated protein tau (MAPT), the gene that encodes tau[36]. In addition to NFTs, neuropil threads (which are the abnormal forms of neurites[37], the processes of a neuron that extend from its soma) are a characteristic lesion in AD and are composed of straight filaments that stain for ubiquitin and tau[38].

Another marker is β-amyloid. Despite being a classic hallmark of AD it has, much like tau, become implicated in being of some pathological significance in PD as well[39]. Extracellular amyloid plaques between neurons are composed of fibrils of proteolytic fragments of amyloid precursor protein (APP). APP is a large, ubiquitous membrane glycoprotein and is cleaved by two proteases. Extracellular cleavage is by an aspartyl protease (β-secretase) whilst intramembranous cleavage is achieved by ɣ-secretase. A peptide fragment of 40-43 amino acids (Aβ) accumulates in fibrils of twisted-paired ribbons in plaques. Microglia are also crucial here; once again, the known mechanisms in AD may also be applicable to PD and associated synucleinopathies. Microglia misinterpret the stacked β-sheets of amyloid extracellularly and ingest them. This damages their lysosomes and leads to regurgitation of amyloid[40], leading to chronic gliosis as a vicious cycle begins. This leaves the area in a state of chronic inflammation. We see that microglia, once activated, lead to bystander cytotoxicity and when partially digested fibril components are released, they form toxic extracellular species (of which nitrogen reactive species (NRSs) are an example) that bind to and damage the plasma membrane of neighbouring cells, leading to bystander killing[41]. The presence of these misfolded proteins leads to downstream effects within the neuron. This includes endoplasmic reticulum (ER) stress, which regulates protein folding through the unfolded protein response (UPR)[42]. One of the arms of this response that deals with misfolded proteins is protein kinase R-like ER kinase (PERK), and when the pathway of this kinase is active for a prolonged period it inhibits the transcription of the pro-survival anti-apoptotic B cell lymphoma 2 (Bcl-2) factor, leading to apoptosis[43]. Like in parkinsonism, genetic polymorphisms can play a role in AD. Apolipoprotein E (ApoE) is the main genetic determinant of risk for AD[44]. Specifically, the APOE ε4 allele increases the risk whilst the APOE ε2 allele is protective and is associated with a decreased risk[45]. Potential treatments for neurodegenerative diseases that involve the clearance of these misfolded proteins are currently being explored. Adipose tissue-derived mesenchymal stem cells (ADSCs) have been found to secrete exosomes carrying neprilysin (NEP)[46], which is the main enzyme involved in the clearance of β-amyloid[47]. The delivery of enzymes that can clean up and process these deposits using cells already present in the body may also pave the way for treatment of synucleinopathies.

After having discussed the roles microglia can play in neuroinflammation and neurodegenerative disease, they are a crucial player to consider. It is clear that if abnormal misfolded and aggregated proteins are being deposited in tissue, the sentinels of the neural immune system, the microglia, will change their behaviour in response to these changes in the homeostatic milieu. Indeed, it has been identified that the aberrant processing of self-proteins (such as α-synuclein) leads to the presentation of their epitopes by the major histocompatibility complex II (MHCII) to T cells that are specific for this epitope and have escaped thymic selection, which in turn

incriminates human leukocyte antigen (HLA) (MHC's gene encoding region) in this damaging autoimmune response[33]. Studies in mice show that $CD4^+$ lymphocytes (T-helper cells) were much higher in number in PD models than in healthy controls, and that they infiltrated the substantia nigra, and were located in vasculature near dopaminergic neurons. Further studies show that a subtype of T-cells (Th17 Lymphocytes) can induce cell death in human induced pluripotent stem cells that model PD[48].

We need to therefore also explore the involvement of HLA, a key and reliable marker of microglia, as the interaction between these resident neural sentinels and their peripherally arriving cousins seems to be a key feature in the pathogenesis of parkinsonism. Interestingly the BBB, usually considered to be the epitome of an immunologically privileged tissue contributes to the increased recruitment of this peripheral T-cells, through its upregulation of the VCAM-1 receptor on the surface of endothelial cells[49]. We can appreciate therefore that synucleinopathies are complex diseases that have a large number of underlying and interconnecting contributors, including protein misfolding, neuroinflammation, vascular morphological changes, peripheral lymphocyte recruitment and microglial phenotype alterations.

These molecular targets are not just used for research purposes, they also play a critical role in the staging of diseases. In this way a neuropathologist can determine the severity of the disease in question. An example of this is Braak staging, where AD severity can be assessed between levels I through to VI based on the presence of markers, such as the hyperphosphorylated tau which we will target[50]. The presence of these markers in different regions of the brain is assessed from entorhinal to limbic to the neocortex and the case is given a stage which reflects how the disease progressed temporally.

Case details:

The patient history is also an important and valuable resource for extracting potential further differences within the cohorts. Knowing the patients' sex, the age of onset of their disease, their age at death as well as the duration of the disease may help elucidate some relevant details for future extended research. Many neurological conditions have differences based on sex, and PD is one of them with men having a higher risk[51]. Factors such as the post-mortem interval (the duration of time in hours between the patient being certified dead and their brain arriving and being treated and prepared at the brain bank) is also an important quality control metric; here we would expect that there are no significant changes. However, this last variable is only a rough estimate, as there are many factors that are taken into account: location of patient death, time taken for autopsy, time taken for removal of brain and time taken for delivery of the brain.

Materials and Methods:

Brain tissue preparation:

The brains first have to be sectioned and sampled for neuropathological examination. In the initial brain sectioning, the brains are sectioned coronally to identify different pathologies that may be present, and the appropriate cases are selected. The selection of the tissue is incredibly important, as this is what can make or break an experiment; our work will be done with this neuropathological diagnosis in mind. Clinical diagnosis and patient history also form part of the framework of case diagnosis and selection. After the brain sectioning, the samples are mounted on a microscope slide at a thickness of 10µm in thickness using a cryostat, which enables a one cell thick layer to be adhered to the slide.

Selection of specimens:

The cohort chosen consisted of seven samples each of PD, PDD and DLB, and five 'healthy' controls (defined by the fact that by the time of death they were not diagnosed with any of the three conditions).

Tissue staining:

The mounted brain samples were stained in a two-day protocol. Cingulate cortex tissue was exposed to a number of antibodies. The primary antibodies used were: Phospho-tau monoclonal antibody AT8 (*Invitrogen*); Purified Mouse Anti-α-Synuclein (*BD*); Purified anti-β-Amyloid 4G8 (*BioLegend*) and HLA-DP-DQ-DR Antigen (*Dako*). (For the detailed protocol see *Appendix A – Detailed Methods*).

First, tissue was covered with 10% normal horse serum which acts as a blocking agent. A blocking buffer is used to prevent nonspecific binding of the antibody to other targets within the tissue.

The primary antibody used is specific for the target, such as HLA or α-synuclein. After an overnight incubation, an ImmPRESS® Horse Anti-Mouse IgG PLUS Polymer Kit (*Vector Laboratories*) was used. ImmPACT™ DAB (*Vector Laboratories*) was used as a stain with Meyer's haematoxylin (*Sigma-Aldrich*) being used as a counterstain.

The tissue was then dehydrated using successive rounds of ethanol and xylene, after which it was cover slipped with DPX mountant (*Sigma-Aldrich*).

Analysis of stained tissue sections:

The stained slides were processed in a high-throughput scanner which prepared high resolution images for analysis. These images were then be used with the HALO pathology imaging platform which has the ability to quantify the levels of brown staining, in this case the brown being the areas of our desired targets.

Two types of software analysis tool were used, Area Quantification and Microglia Analysis. The former detects the percentage of the tissue that is stained brown (using the optimised parameters) whilst the latter detects microglia within the tissue (once again instructed by parameters) and provides information on important features such as activation percentage and the thickness of the area taken up by microglial processes (Figure **1**).

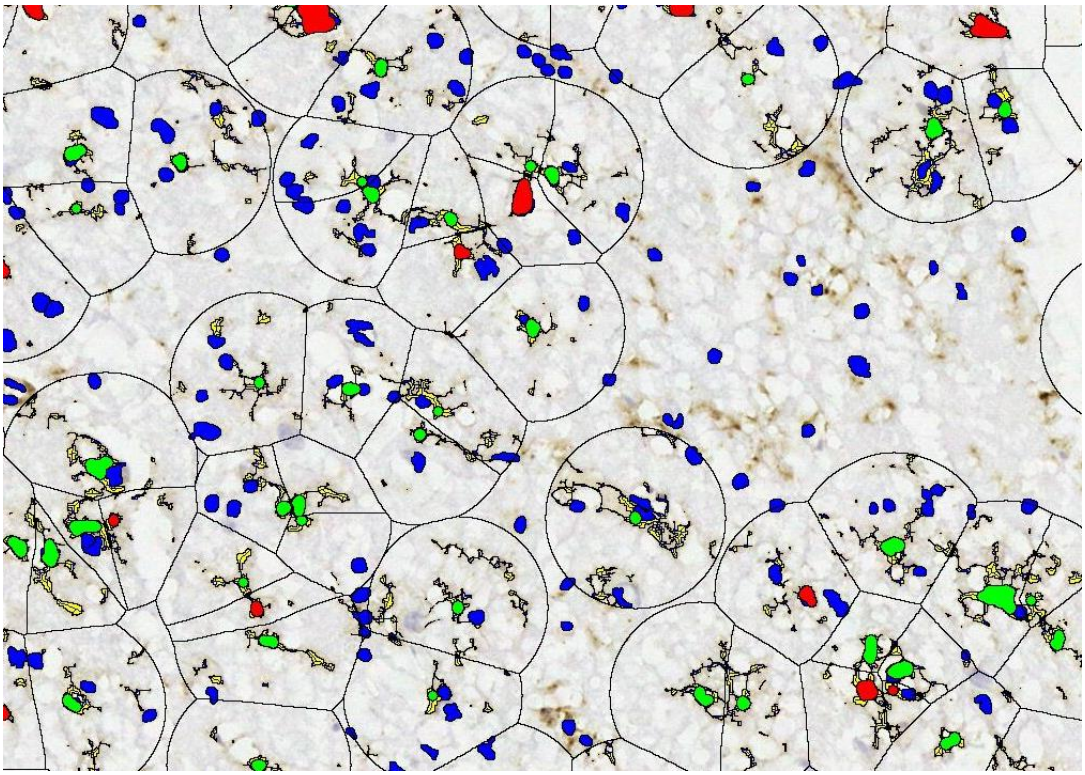

*Figure 1: Microglial analysis software. Nuclei are highlighted in blue. Microglia that are activated are highlighted in red, whilst inactivated ones are in green. The black borders represent the borders of the microglial cells' sphere of influence, which is here defined by where the processes (in yellow) of that microglial cell are extending. Note the middle-right of the slide; there **is** brown (DAB) staining but it is not being included in this particular analysis. This is because the tissue is taken as a single thin section and those are processes from microglial cells that were either 'in front of' or 'behind' this particular tissue sample when it was one block.*

Results:

In area quantification analysis, we are looking for the percentage of the grey matter in the tissue section that is stained brown for the desired target (Figure **2**).

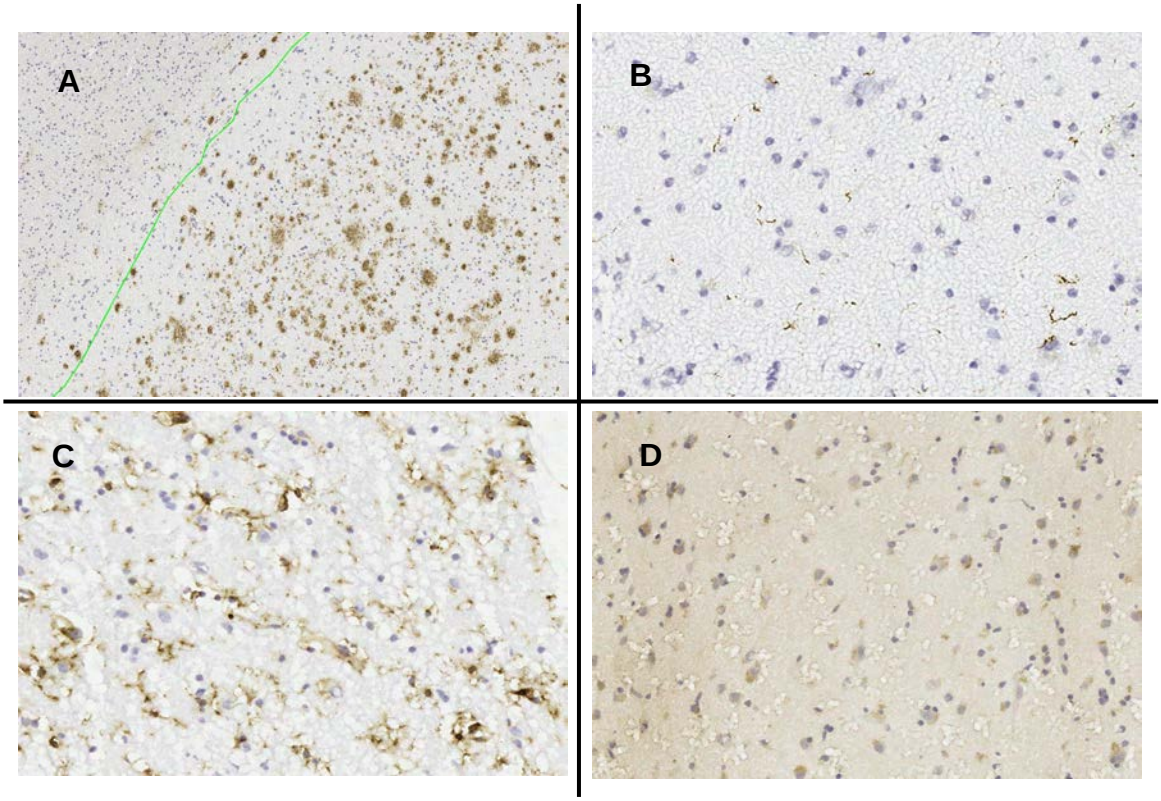

*Figure 2: Representative images of the different types of staining. Stained brown in each picture are (**A**) β-amyloid plaques; (**B**) tau tangles; (**C**) HLA staining; (**D**) α-synuclein aggregated in Lewy bodies around nuclei. Nuclei can be seen in blue. Note the green line top left represents the boundary of analysis (separating white matter and grey matter).*

From the area quantification analysis, we can conclude that the percentage of grey matter in the cingulate cortex stained for HLA was greater in cases with PD in comparison to those with DLB (Figure **3A**). PD cases had, on average, 2.495% of the tissue stained for HLA in contrast to DLB cases which had 1.238% of the tissue stained and this difference is statistically significant ($p = 0.0426$). We can also conclude that cases of PD had a higher percentage of tissue stained for HLA than the 'healthy' controls ($p = 0.0252$).

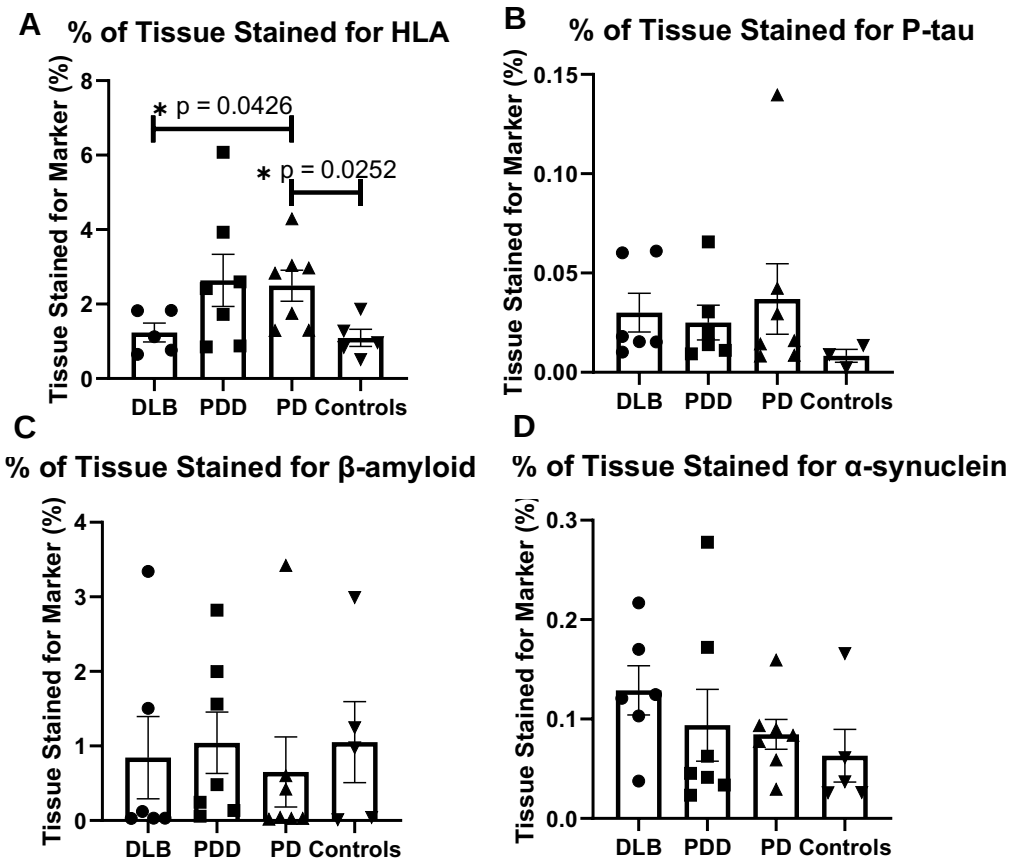

*Figure 3: The percentage of tissue stained for the different markers. (**A**) HLA (**B**) P-tau (**C**) β-amyloid (**D**) α-synuclein. In (**A-D**) there are four groups: dementia with Lewy bodies (DLB) cohort, Parkinson's disease dementia (PDD), Parkinson's disease (PD) and 'healthy' controls (Controls).*

The other comparisons have found no other statistically significant differences in the marker presence between the different disease cohorts and no significant correlations with regard to age at death, age at onset, duration of disease or PMI.

Each of the properties of microglia that were measured provide us with a small insight into their activity, allowing us to make inferences regarding their phenotype. The first measurement is the number of microglia that are activated. Activated microglia are those that are pro-inflammatory (M_1-like phenotype) and are seen as being a hallmark of neuronal pathology which involves inflammation of the tissue[52]. However, as mentioned before this is an oversimplification and as one article notes that this may be an "outdated terminology since microglia are known to constitute a heterogeneous cell population that undergoes highly adaptive changes in both morphology and function depending on the context of health or disease[53]." We can also consider their processes, particularly their length and thickness. The process area refers to the area covered by processes that extend from a microglial cell, whilst the process length is a measure of their length as they extend from the soma out to the tissue.

When looking at the microglial properties and how they vary across the different disease states (Figure **4**), a number of observations can be made. We first note that microglial activation was very similar between the different cohorts (Figure **4A**). Albeit not statistically significant, we notice a trend where the average process area *per cell* (Figure **4B**) does not vary whilst the *total* process area has a stronger trend, with DLB and Control cohorts having a lower value (Figure **4E**) in comparison to PDD and PD which have a mean value that is more than doubled. A similar trend is seen with the average process length *per cell* (Figure **4C**) in comparison to the *total* process length across the whole tissue section (Figure **4D**).

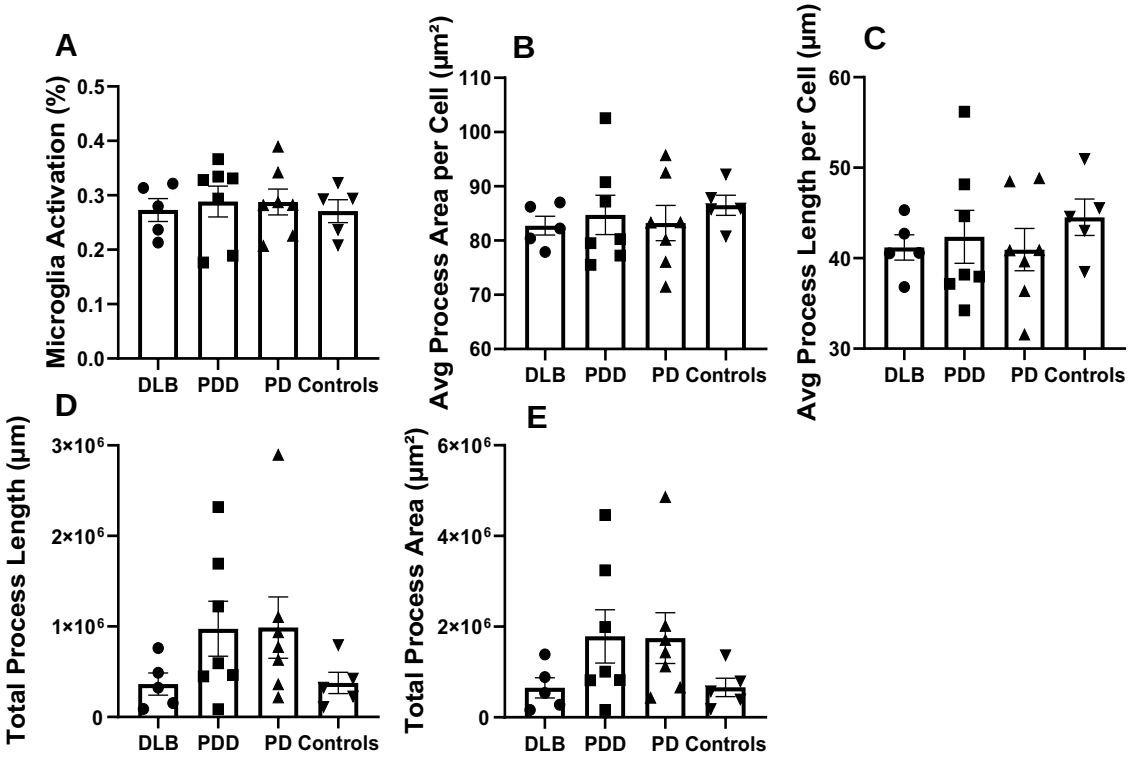

*Figure 4 – Different microglial properties and how they differ in disease states. (**A**) The microglia that are activated as a percentage of all microglia in the analysed tissue section. (**B**) The average process area per microglial cell. (**C**) The average process length per microglial cell. (**D**) The total process length and (**E**) the total process area as a sum of all of the microglial processes in the analysed tissue section.*

When looking at how the samples vary when compared by their clinical details (such as sex and duration of disease) we found consistent trends in our data. When looking at the effect of sex on the percentage of the tissue stained for the different markers, the data is much more variable in the male cohort than the female cohort. Furthermore, the effect of sex on the microglial properties is varied (Figure **5**). Female samples in the cohort had a higher percentage of their microglia activated (Figure **5A**), however not yet statistically significant. Consider that, as previously indicated, this data may be influenced by confounding variables stemming from the heterogeneity of microglial phenotypes in tissue. Interestingly, the average process length and area *per cell* are very similar between male and female cohorts, whilst there is evidence of a lower total process length and lower total process area in males.

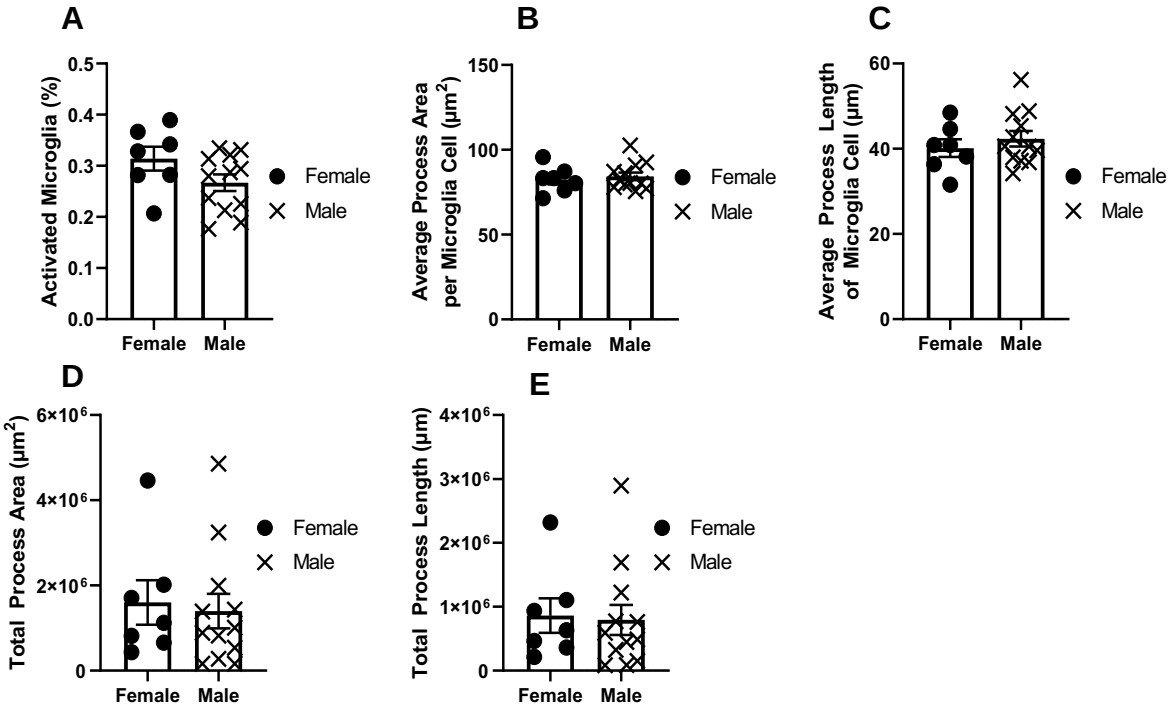

*Figure 5 - The effect of sex on microglial properties in brain tissue. (**A**) The microglia that are activated as a percentage of all microglia in the analysed tissue section. (**B**) The average process area per microglial cell. (**C**) The average process length per microglial cell. (**D**) The total process length and (**E**) the total process area as a sum of all of the microglial processes in the analysed tissue section.*

When considering the effect of the age of onset of the disease on the microglial properties, we notice that microglial activation does not visibly vary (Figure **6A**), however we see that the earlier the disease begins in a patient's life, the other four parameters of microglia all tend to be slightly lower (Figure **6B-E**).

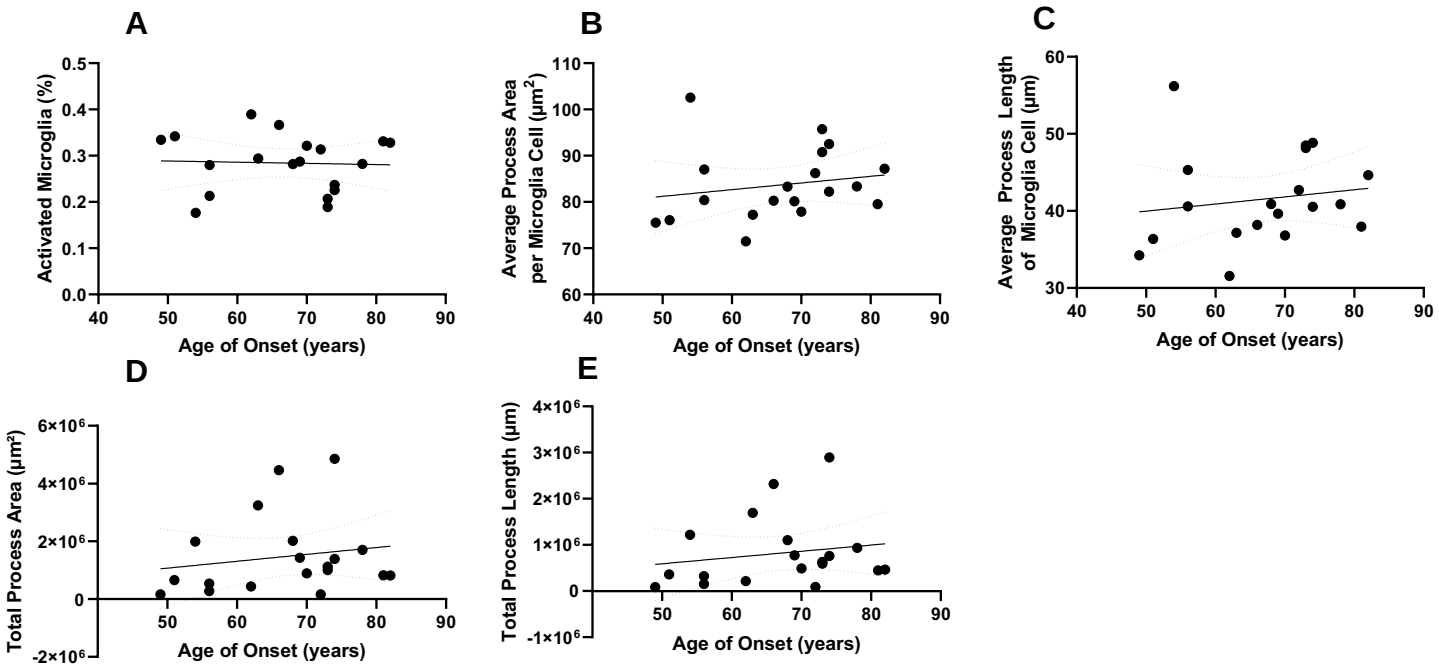

*Figure 6 - The effect of the age of onset of a synucleinopathy on microglial properties in brain tissue. (**A**) The microglia that are activated as a percentage of all microglia in the analysed tissue section. (**B**) The average process area per microglial cell. (**C**) The average process length per microglial cell. (**D**) The total process length and (**E**) the total process area as a sum of all of the microglial processes in the analysed tissue section.*

For the remainder of the data please see *Appendix B – Detailed Results*.

Discussion:

In a typical healthy brain the soma of a microglial cell is small with long processes that branch extensively and can extend far beyond what we would expect to be the spatial remit of the cell[54]. On the other hand, microglia that have been found in disease states such as AD[55], Huntington's disease[56] as well as acute brain injury[57] have microglia that are shorter and thicker. It is however also important to consider that we can't make all the conclusions from just studying the morphology of these cells. They are incredibly varied in their transcription factor production, their transcriptomics profile and thus in the expression of genes and production of proteins. Thus, we are only seeing a part of a picture, onto which data drawn from other methods (such as RNA-Seq) will need to be superimposed on.

The fact that the microglial properties vary in some respects but do not in others tells us something about the different immunological milieu in different disease states. For example, targeted studies in mice using human α-synuclein to model PD have found that it upregulates the production of the pro-inflammatory cytokine IL-1β[58]. On the other hand, studies suggest that in DLB, there is an upregulation of two different cytokines, IL-1α and TNF[59]. In our results, the fact that the values *per cell* are quite similar but the total values of microglial process area and length are lower in the DLB cohort than PDD or PD suggests that DLB brains have fewer microglia infiltrating their cingulate cortex, however the extent to which those microglia are 'activated' is similar. Moving forward, the percentage of tissue stained for HLA is higher in PD brains than DLB brains. When we compound this significant difference with the suggestions from the findings of the microglial analysis, we can with higher certainty say that the cingulate cortex of patients with DLB has a lower microglial presence overall, but those microglia that are resident in the tissue are of a similar phenotype to those in PD (and even PDD). Other studies however found (using PET scans and cytokine analysis) that in comparison to healthy controls, DLB patients have increased peripheral inflammation and early microglial activation in areas affected by DLB pathology[60]. From our data there is some suggestion that PDD cases have, on average, a higher process area and one could argue that this is due to more extensive branching. However, when compared with the microglial activation values, the PDD cases maintain a higher average; thus, suggesting that in PDD the arbours could be thicker and in fact suggest a more pathological phenotype. Microglia vary in their structure in different pathological states. These range from the ramified microglia with long and thin processes that are in the 'resting state', followed by intermediate microglia which have shorter and thicker arbours and on the other end of the spectrum to amoeboid microglia, which have lost almost any visible processes and have an enlarged soma in response to an insult to the brain region in which they find themselves[61].

Something similar can be deduced when looking at the effect of sex on microglial properties. As the average values per cell are once again similar, yet the total process area and length are higher in females on average, we can speculate that females have a higher microglial presence in the cingulate cortex. An interesting study on the differences in microglia between male and female adult mice may shed light into an explanation for this difference. It concluded, using cell culture and female-to-male microglial transplantation, that the microglial transcriptome is sex-specific in its

expression and this is affected and maintained by circulating sex steroids[62]. They also suggest that the microglia of female mice is more neuroprotective as the damage caused by a localised acute cerebral ischaemia is restricted.

The effect of the age of onset of the disease is an interesting one to consider as one would assume that a disease process that is part of a chronic degenerative decline would be of a more aggressive phenotype in cases of sporadic diseases that initiate earlier on in the patient's life. The data suggests that patients whose disease began at an earlier stage in their life have a lower microglial average process area and length. This suggestion is also presented in a study that showed that non-aggregated α-synuclein can induce a pro-inflammatory microglial phenotype through TLR signalling[63]. Perhaps this could contribute to more early-onset forms of synucleinopathies. The microglia of these patients could be of a more pro-inflammatory phenotype with shorter and thicker ramifications to start with. We can speculate that one of the contributing factors to a more aggressive phenotype that affects a younger population will be linked to the neuroinflammatory environment and its regulation (or lack thereof) by microglia. Indeed, there are studies that suggest that there are polymorphisms in genes coding for pro-inflammatory markers that are generally elevated in PD. Examples include TNF-α[64], IL-6[65] and IL-1β[66]. Interestingly, PK11195 (a marker of microglial activation) was used in conjunction with PET scanning to deduce that healthy controls had less inflammation than DLB cases, but greater inflammation than late stage more severe DLB patients[60], which suggests a non-linear inflammatory profile change, something that needs to be taken into account in the future with larger cohort studies.

Limitations of the study and potential improvements:

There are a number of aspects of this preliminary study which could have impacted its ability to determine differences with higher certainty. Firstly, the power of the study. In each disease group n=7 and in the control group n=5, giving a total of 26 samples. These results then, particularly those that showed signs of potential difference, may be able to serve as a guide in the future for larger studies with larger cohorts. Indeed, as a systematic review reports[67], there is large variation in the reported cases of PD and PDD across different studies, with some studies finding that about 15% of PD patients have symptoms of dementia[68], whilst some concluding that the number may be as high as 80%[69]. This pointed to a need for further clinical refinement for PDD and DLB in the diagnostic criteria, such as that carried out by Dickson et al. in their neuropathological analysis of PD[70].

Further decreasing the power of the study, some samples had to be excluded. Despite most of the staining being clear and specific (a testament to the specificity of the antibodies we used), there was some background staining in a handful of samples. This was due to tissue being damaged and torn, leading to DAB accumulating in the areas and producing a strong brown signal that was not in fact specific for the target. There are a number of reasons this type of damage may occur, including the way the tissue is processed. The samples must be mounted on a microscope slide at a thickness of 10µm. This is a delicate procedure and when combined with the fact that

even something as simple as ice crystals making their way onto the tissue can damage it in the long term, some samples were more damaged than others.

Despite the fact that this project looks at synucleinopathies from a molecular perspective, clinical data is invaluable, however not always perfectly reliable. One of the first brains we prepared illustrated this difficulty. This particular case was an elderly male that was diagnosed with PD. On inspection of the midbrain, there certainly was decolouration of the substantia nigra at the level of the oculomotor nerve (consistent with nigrostriatal dopaminergic neuron loss), however when inspecting the coronal sections there was atrophy and necrosis of the putamen, and this was quite extensive. Thus, there is a chance that there was a misdiagnosis by the neurologist or psychiatrist and this patient actually had a similar condition called multiple systems atrophy (MSA). MSA is a rare sporadic movement disorder that also has degeneration in the striatonigral pathway with glial and neuronal inclusions that are positive for α-synuclein. However, Lewy body pathology is absent in this case and we wouldn't classify this as a Lewy body disease[71,72]. Through this case one can see that there are a number of differential diagnoses that need to be ruled out when identifying PD pathology. Examples include MSA, corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP). This example further illustrates that the complexity of these diseases (as well as their elusiveness) is not just present in the biochemical understanding of their causative factors but also the clinical management and diagnosis of patients.

Another aspect which makes reaching conclusions more difficult is that the tissue that we are working with is a snapshot of one person's brain condition at one point in time, and this does not necessarily tell us the whole story. For example, when comparing PDD and DLB, the former begins with PD symptoms and dementia arises later in life whilst patients with DLB experience cognitive decline first with a slower progressive akinetic phenotype developing later on in the disease. This is something we just can't track with a brain tissue section as we only see the terminal neuropathological state. When researching other diseases such as AD, there are useful approaches we can utilise to mitigate this problem. In the lab we also extracted DNA from brain tissue of AD patients. Here we examined three areas: the entorhinal cortex, the medial temporal cortex and the somatosensory cortex (consider the aforementioned Braak staging). In this order they represent the usual regional progression of the disease. Thus, even with the terminal snapshot that is the brain section we were able to at least simulate how the disease changed over time, something that was not possible in this project.

Another point to note is the part of the brain that was sampled for testing. This was the cingulate cortex. This is not a typical area of pathology for these diseases, as that would mostly be the basal ganglia[73]. The 'terminal neuropathological snapshot' that we examined therefore was not only a temporal one, but also a regional one. This is an issue because it would be incorrect to assume that a single area is representative of the whole brain. Indeed, studies in mice have confirmed that there are differences in the microglial distribution between different brain regions in the healthy brain[74], and we can thus speculate that this may underlie the different regional levels of microgliosis[75] (that is the increased pro-inflammatory state microglia develop when faced with pathological surroundings[76]) in a disease state. It is important to note

however that the basal ganglia are not the only ones affected in PD-associated diseases, as the thalamocortical[77], corticobulbar and corticospinal pathways are also affected, and the latter two are so implicated due to their importance when localising electrode placement for deep-brain stimulation surgery procedures[78]. Thus, there are benefits of examining other areas apart from the midbrain and the substantia nigra pars compacta (SN_{pc}) as it is clear that the patient experience of these diseases is more complex than solely motor symptoms.

Another area of this study to consider when evaluating our results is the procedural side. In order to pick up as much of the DAB staining as possible (without including background staining, debris, tissue folding artefacts etc…), it is imperative to select the correct parameters for the data analysis. In this case, in each staining cohort, we found the samples with the least signal (most faintly stained brown for DAB) and samples which high signal (darkest staining), and adjusted the min/max thresholds so that values that fell within these borders could be accommodated in the computer analysis and picked up. A compromise always has to be made with this type of data collection.

We focused on grey matter in this project because that tissue has been used in other areas of the lab in previous experiments such as in RNA-Seq. RNA-Seq is a novel technology that allows the study of the RNA profile of a particular cell within the brain and also how it changes over time over different cell types[79]. This used to be hard to do as RNA is not as stable as DNA and when the tissue was freeze-thawed the cells would burst leading to loss of RNA. However, with this modern technique it is now possible to isolate the nuclei and thus have an accurate representation of the transcriptomic profile of the cell. In the interests of consistency, the area (grey matter) analysed in other accompanying projects was kept the same in this project too.

Furthermore, one of the challenges of the image analysis is that the software cannot differentiate between different types of tissue. For example, it cannot detect whether to analyse only grey matter, and so manual annotation had to be carried out on each tissue sample to select for only the grey matter for analysis (*Appendix C – Tissue Images*).

Conclusion:

The present findings show that the percentage of tissue stained for the microglial marker HLA is higher in patients with PD in comparison to those with DLB. Correlating these findings with genetic and transcriptomic profile studies can help us elucidate new mechanisms that underscore the heterogenous clinical profiles of synucleinopathies. Despite intensive experimentation into the nature of neurodegenerative diseases, some are still elusive, both at the bedside and the bench. To help strengthen these findings, we might need to look elsewhere, more specifically to rare genetic diseases. These contain neurofilament aggregation as a main feature and include giant axon neuropathy and Charcot-Marie-Tooth sydromes[80]. Thus, larger scale studies comparing the synucleinopathies, compounded with these relatively newly discovered Mendelian disorders could enable us to more closely understand the roles of protein misfolding, aggregation and failure of clearance that are the key yet elusive feature of chronic degenerative diseases.

Acknowledgments:

In my time at the Dementia Research Institute I had the opportunity to both contribute and learn about different aspects of the lab work carried out in the group, as well as carrying out my own research project with a specific aim and data collection. I learned about the different ways we can investigate neurodegenerative diseases, including new methods which I have never been exposed to before such as RNA-seq and bulk RNA analysis. This specific research project arose after some weeks learning and assisting with the different experiments being carried out in the lab. The ultimate goal is to understand and specify which markers correspond with which neuropathological state, and further correlate this with the different ‘omics profile of the patient, their genetic background as well as the patient history.

I would like to thank everyone who taught and guided me during my time in the lab. It was immensely invaluable for me to be able to participate in research regarding a group of diseases that I want to focus on in my clinical practice further down the line. I am particularly thankful to Dr Amy Smith and Karen Davey who taught me everything and showed me how everything works in the lab, Professor Paul Matthews who took me onboard to join his lab as well as Professor Steve Gentleman who allowed me to observe the preparation of tissue at the brain bank. I am also very thankful to Merton College for enabling me to carry out this research by awarding me the summer research project grant.

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Supplementary Material:

Appendix A – Detailed Methods:

Tissue staining protocol:

Day 1:

Sometimes, tissue can be fixed with PFA or formalin, however here we chose to use fresh-frozen tissue. There is an advantage to the alteration of using methanol for a brief and weak fixation instead of PFA or formalin. This is for a number of reasons: PFA damages tissue slightly; all other work (such as RNAseq) has been performed on these cryostated fresh-frozen sections; some antibodies don't bind well to more-heavily fixed tissue, and additionally there is no antigen retrieval needed.

1. Remove slides from -80ºC freezer.
2. Submerge in 100% cold (4ºC) methanol for 6 minutes
3. Rinse in PBS, 3x5 min.
4. Eliminate the endogenous peroxidase activity with 0.3% H_2O_2 in 1xPBS for 30 min in dark.
    a. (3mL of H_2O_2 made up to 300mL)
    b. Cover with black tray.
5. Rinse in PBS, 3x5 min.
6. Incubate sections in 10% normal horse serum made up with PBS for 1h at room temperature - blocking.
    a. 5.4mL total with 4860µL of PBS and 540µL of horse serum, invert mix
    b. Dry edges of slide with tissue, and annotate with PAP pen
    c. Add 200µL of mixture to each slide, ensuring entire tissue is covered
7. Incubate sections with 200µL primary antibody (see below for dilutions and values) in PBS, overnight at 4ºC.
    a. Tap-off serum

Day 2:

Note that normally the procedure would be to use a biotinylated secondary antibody, and then using a tertiary mixture such as the Vectastain Elite ABC kit to bind to the biotin and enhance the strength and accuracy of the staining. However, newer kits have been developed where these two steps are combined, giving both a simplified and a quicker incubation period. In this case, as we are using a mouse antibody with normal horse serum as the blocking agent, we will be using a horse anti-mouse kit called ImmPRESS® Horse Anti-Mouse IgG PLUS Polymer Kit.

1. Rinse in PBS, 3x5 min using a squirt bottle.
2. Incubate each slide with two drops of the ImmPRESS mix for 30 minutes (covered).
3. Rinse in PBS, 3x5 min using a squirt bottle.
4. Make up DAB mixture (1 drop of DAB with 1mL of diluent)
    a. In this case make 6mL and 6 drops using the ImmPACT™ DAB.

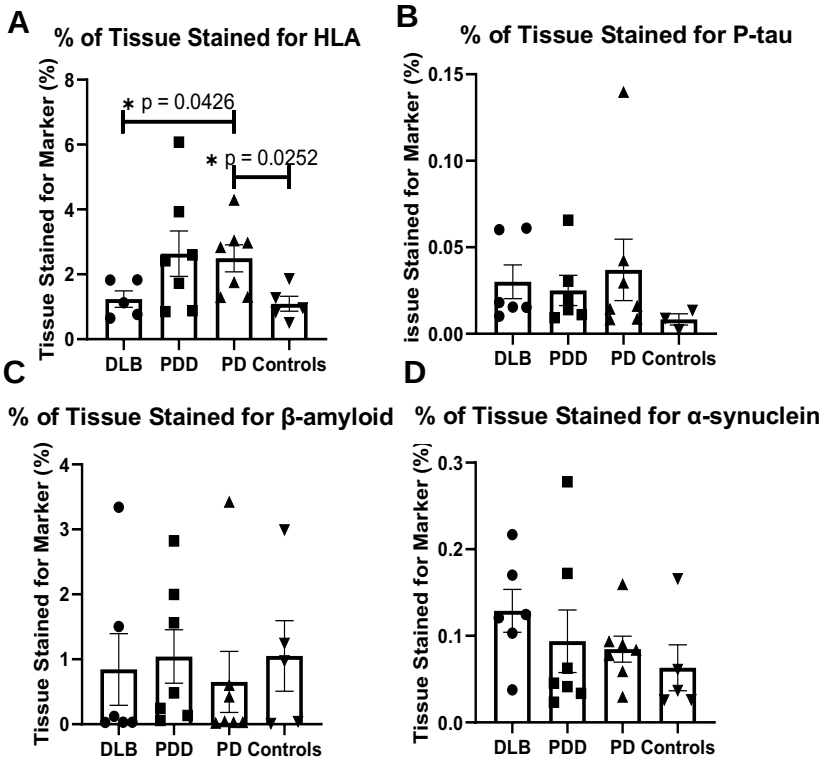
5. Add 200µL of the DAB mixture to each slide, leave for 1 minute, tap off and then spray with PBS.
6. After doing one row, load in a rack placed in a trough of distilled water.
7. Place under running water for 2-5 minutes.
8. Check staining under microscope.
9. Counterstain with Haematoxylin (Meyer's) for 1 min.
10. Rinse sections in tap water for 5 min.
    a. This is necessary for the blueing reaction; it has to be tap water.
11. Check for counterstaining under microscope.
12. Dehydrate though series of ethanol for 5 minutes each (75%, 95%, 2 x 100%)
13. Clear in Xylene (1->2->3 in fume hood) for 5 minutes in each
14. Coverslip with DPX.
15. Antigen-expressing cells will be stained brown and cell nuclei are stained blue.

Antibody Calculations:

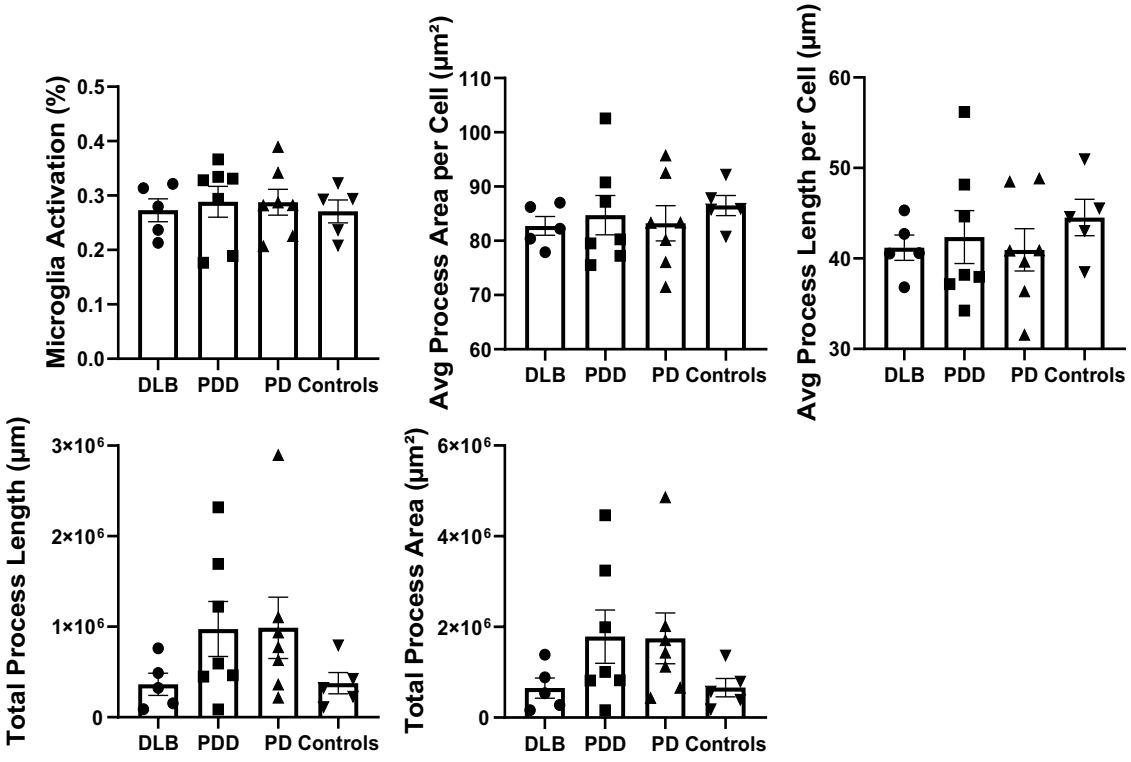
Antibody Target	**Type**	**Dilution**	**Volume of Ab (µL)**	**Volume of PBS (µL)**
P-tau	AT8	1:2000	2.8	5597.2
α-synuclein	RUO	1:500	11.2	5588.8
β-amyloid	4G8	1:1500	3.73	5596.3
HLA	CR3/43	1:500	11.2	5588.8

Appendix B – Detailed Results:

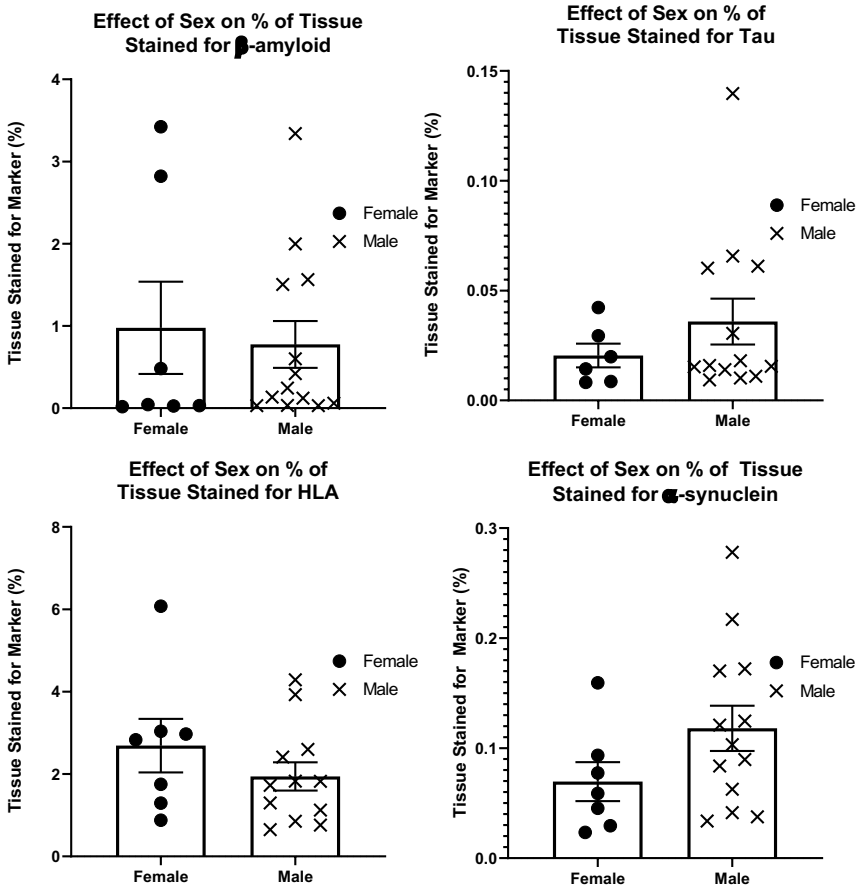
The percentage of tissue stained for markers in the brains of different disease states:



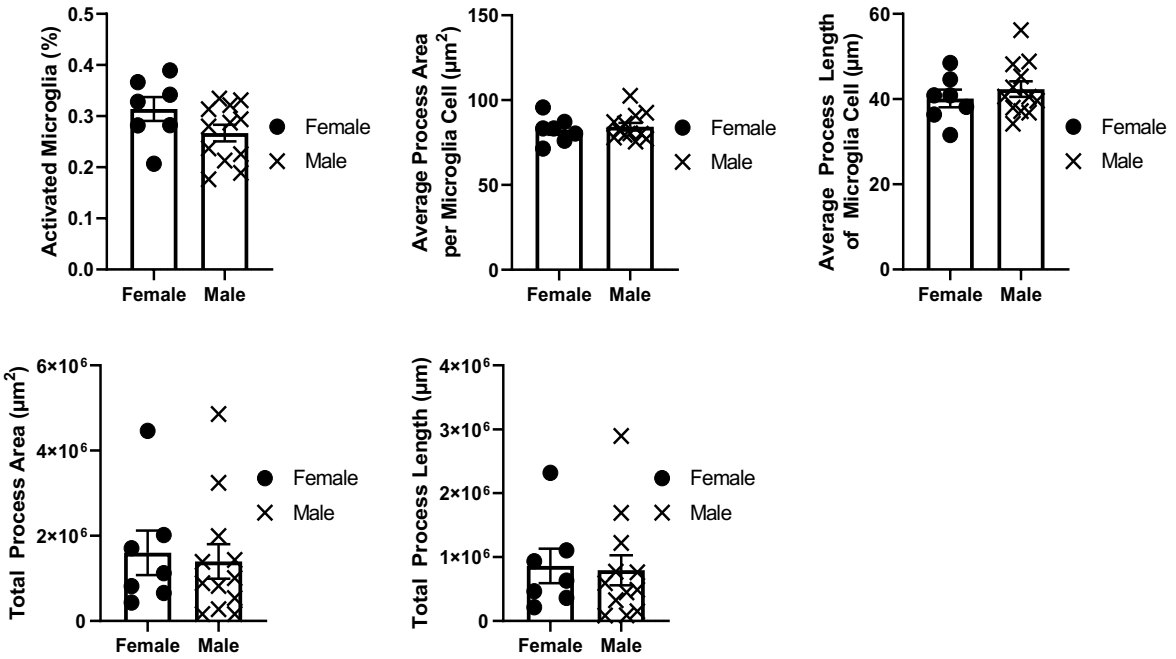
Microglial properties and how they are affected in different disease states:



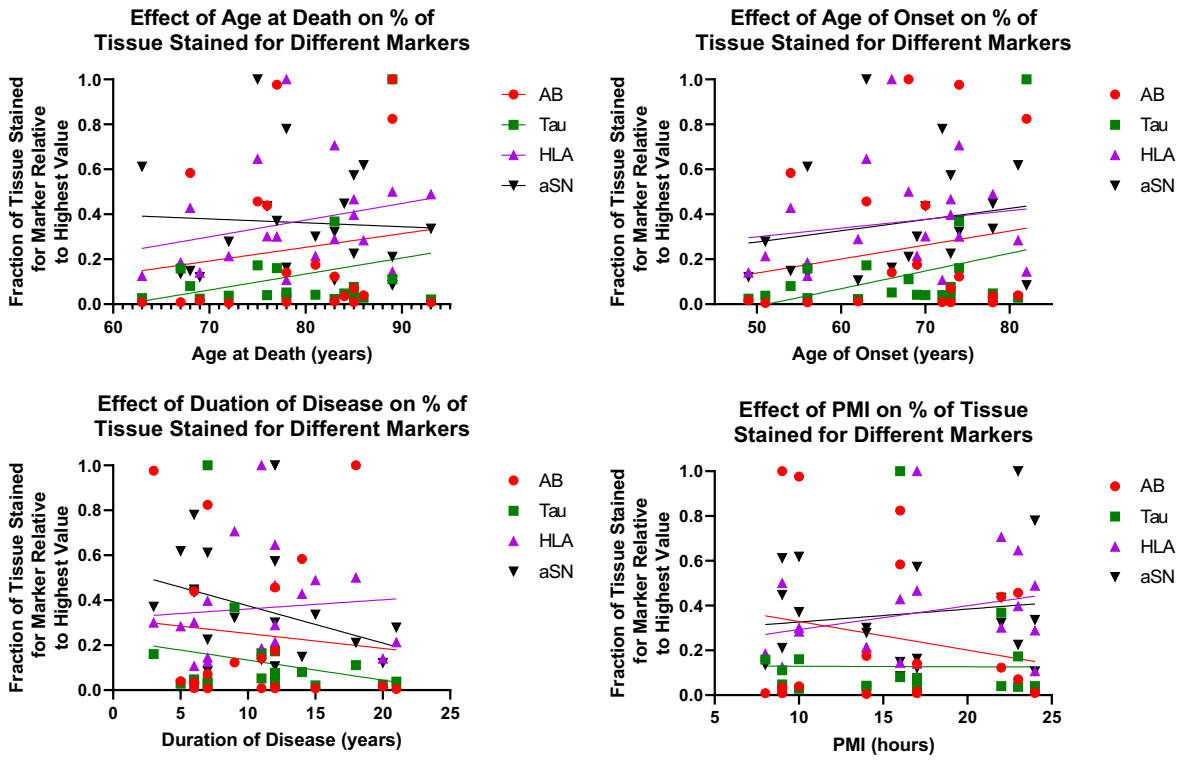
The effect of sex on the percentage of tissue stained for different markers:



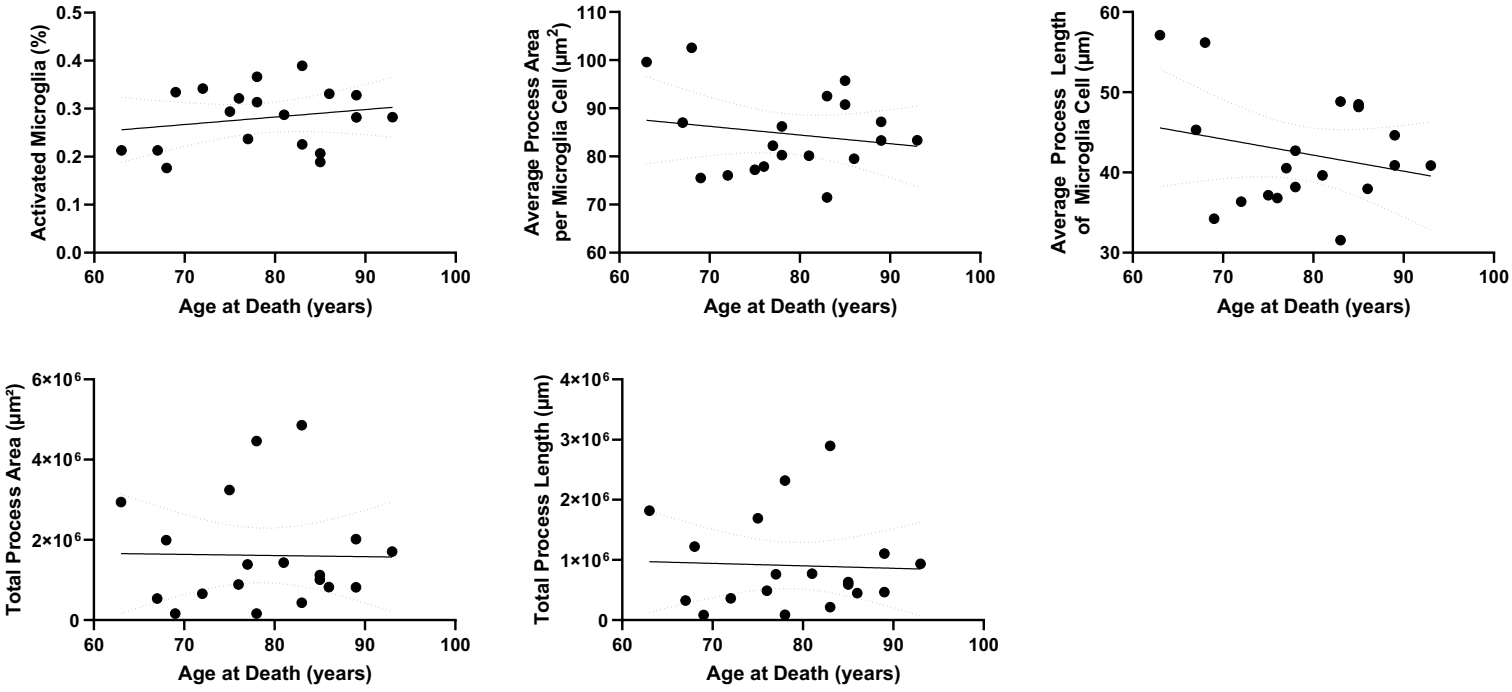
The effect of sex on microglial properties in brain tissue:



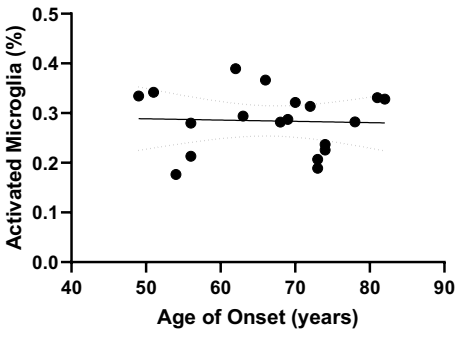
The effect of age of death, onset of disease, duration of disease and PMI on the percentage of brain tissue stained for different markers:

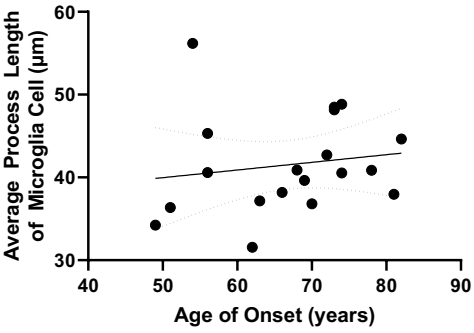


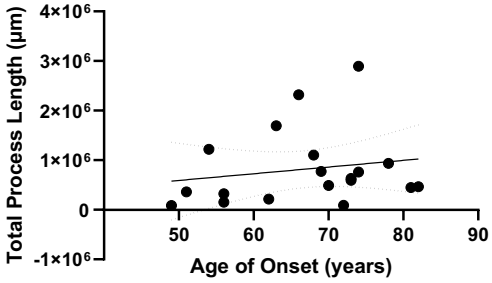
The effect of age of death on microglial properties in brain tissue:



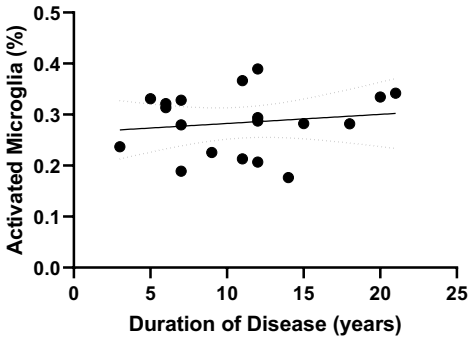
The effect of the age at onset of disease on microglial properties in brain tissue:

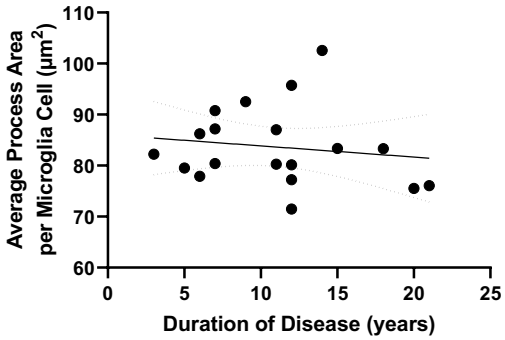


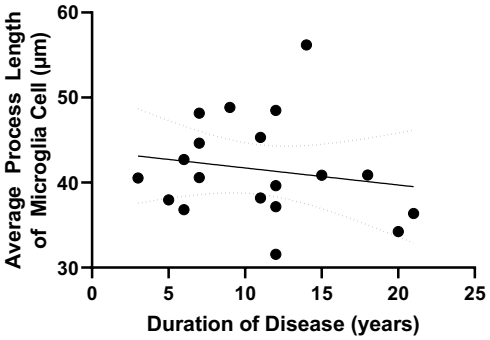


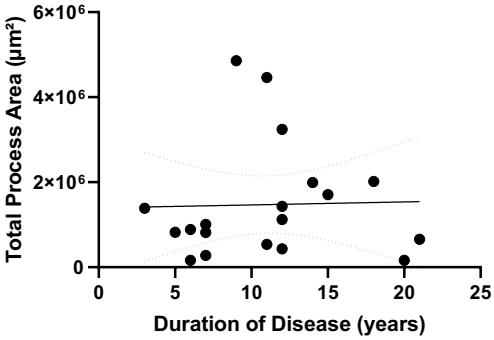


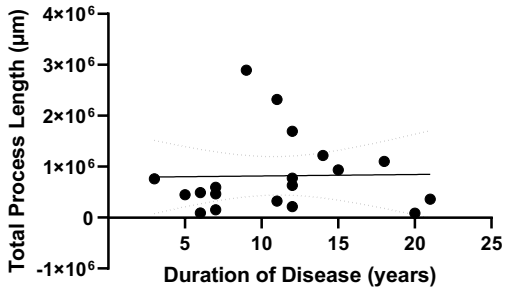
The effect of the duration of the disease on microglial properties in brain tissue:



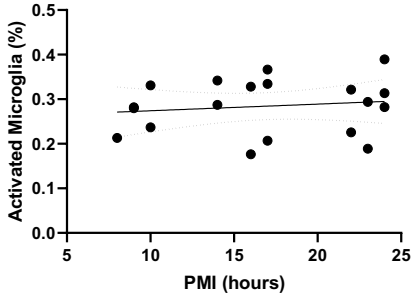


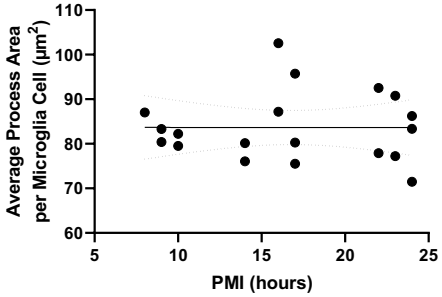


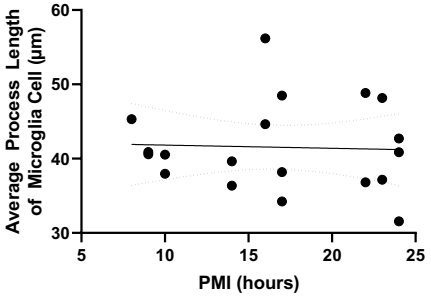


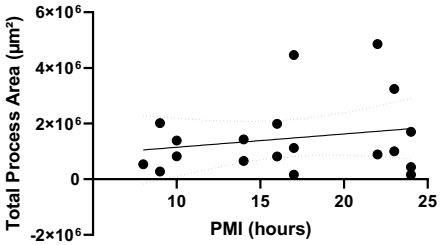


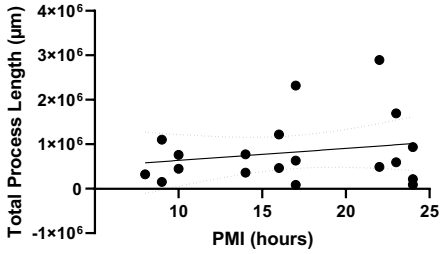
The effect of the post-mortem interval on microglial properties in brain tissue:











Appendix C – Tissue Images:

The difference between grey matter (upper) and white matter (lower), separated by the annotated orange border. Note the density of nuclei in the white matter.