Vascular Parkinsonism (VaP) and the Ageing Brain: Re-examining an Elusive Entity

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1.0 Introduction

1.1 Epidemiology of Ageing

The world is changing, and with it the demography of the human population. In the 2015 World Health Organisation (WHO) report on Ageing and Health, the Director-general hailed these changes as significant and with profound implications. The average global life expectancy at birth, today, is 71.0 years. In the UK and EU, life expectancy is higher, at an average of 83.5 years and will continue to rise. As the post-world war 2 "Baby Boomer" generation now starts to reach an elderly stage of life, the western world faces a larger ageing population. In Britain alone, the number of individuals aged 65 years and over has risen from 8.5m to 10.6m over the past 20 years. Furthermore, the growth in populations of over 85 years is projected to rise by 15% between 2015 and 2020. The UK is home to the largest population of elderly people in the EU. It is a delight that people are able to see more of life, but, this scenario presents unique challenges to healthcare and modern medicine.[1,2,3]

Increased lifespan is influenced by both physical and mental, health and well-being. It is positive to both the individual and society if additional years at an older age are spent in good health. Vice versa, poor health would reflect negatively on the quality of later life. The WHO claims there is little evidence to support that people experience better health now compared to previous generations of similar age. Although people live much longer, it is uncertain whether the quality of additional years has indeed improved. Similarly, the elementary assumption of linking older age to poor health and increased healthcare needs to greater cost and expenditure is one that is unfounded. Evidence shows that the relationship between an ageing population and healthcare expenditure is variable and uncertain. This hypothesis is influenced by local healthcare infrastructure and differences in

service provision. In absolute comparison, the greatest healthcare costs are consistent with the final years of survival. Literature further clarifies that such costs are lower in elderly patients than their younger counterparts. However, it is certain that a growing ageing population is an important stressor on our current system of healthcare. Instead, requiring a rethinking of both health and social care services. This requires a greater appreciation of ageing as an inevitable phenomenon of normal biology.[3,4]

1.2 The Process of Ageing

Ageing or senescence, is the process of living decline due to the time-dependent accumulation of various molecular and cellular damages. It is the most complex occurrence of phenotypic remodelling due to the combined expression of deleterious determinants of health. Such determinants contribute to health biodiversity, presenting through the individuals' genome, the environment and ones choice of positive and negative behaviours.[1,5,6] We can also perhaps think of ageing as the likelihood of encountering death.[7] Even though, death is presently an inevitable endpoint, the degree in health decline may not be directly reflected as a deterioration of well-being. Older age can be a period of an improved state of life. Though this is highly subjective, there is a considerable combination of psychosocial factors which can influence the perception of age.[5,6,7]

The process of reaching death is easily dictated by two mathematical variables, the initial mortality rate (IMR) and the mortality rate doubling time (MRDT).[5,8,9] The former dictates a baseline rate of mortality independent of the impact ageing, the latter is a doubling of the probability of mortality at a specific time. The human population has an average MRDT value of 8 years post the age of 30 years, translating into a doubled likelihood of dying every 8 years after reaching ones 30th birthday![9,10]

Though the MRDT is constant, the IMR is subject to change. In fact, the IMR has improved so drastically, that the adult life expectancy in the UK has increased by about 30 years, over the past century. This improvement in life expectancy and the subsequent drop in rates of mortality is attributed to advances in medicine, surgery and quality of life. In time, increases in human lifespan would inevitably taper off and reach a plateau. At this point, efforts to improve longevity must focus on slowing the rate of ageing itself. This requires an understanding of the physiological and pathological changes that occur throughout life after maturity.[9,10]

1.3 Ageing as an Inflammatory Process

The effects of ageing are numerous and can be broadly characterised as a decline in function, physique and fertility with the expansion of susceptibilities leading to age-related diseases.[11,12] Immunosenescence, brought on by changes to the immune system, is of great interest; manifesting as the gradual deficiencies of both the innate and acquired immune functions.[9,13] It is not a mere decline, instead a process of maladaptive remodelling resulting in an alteration of normal immune homeostasis.[12,13,14] Some immune functions decline, some remain unchanged and some improve. This process is mirrored by how elderly individuals are affected by disease. Immune memory development declines, resulting in poorer response to infections and uptake of vaccines. Meanwhile, diseases such as asthma tend to decline with age. The common cold on the other hand affects all people seasonally, regardless of age.

Franceschi highlights a state of chronic low-level baseline inflammation as a hallmark of ageing, while Vasto defines this pro-inflammatory condition as a 2-4 fold increase in serum cytokines and acute phase proteins such as IL-6, TNF-α and CRP.[12,15] Cardiovascular disease, cerebrovascular disease, atherosclerosis, diabetes and neurodegenerative pathologies are all mutually inclusive, correlating with each other, resulting in neurological dysfunction

of an immunosenescent aetiology.[12,13,14] Most importantly, this pro-inflammatory state is predictive of ageing phenotypes, of which poor neuronal health is imminent and prevalent.[15]

1.4 Cardiovascular and Cerebrovascular Pathology

Cardiovascular disease is prominent in the developed world, accounting for 40% of total deaths.[16,17,18] Although historically associated with well-defined risk factors, habits such as smoking, hypercholesterolaemia and hypertension, fail to account for a significant margin of cases free from such risks.[19] We can consider atherosclerosis as a classical mechanism or cardiovascular disease. As a dysfunction of normal arterial function and structure, it was once thought to be a lipid-storage disease.[20] We now understand that the upregulation of IL-1β underlies hypertension.[17] A pro-inflammatory state alters the haematopoietic profile favouring a pro-coagulative condition[20] while the combined action of IL-6, IL-1β and TNF-α causes endothelial activation and arterial vessel vasoconstriction. Such immune-mediated changes are causative of atherosclerosis while contributing to the pathogenesis of further heart and brain diseases.[16,19]

An elementary example of an acute neurological disease from vessel dysfunction would be stroke. As the second leading cause of death, stroke is a prominent cause of physical and mental disability and impediment globally. As coronary vessel disease would result in myocardial infarction, the same pathology within the cerebral circulation would contribute to cerebrovascular injury. This is commonly seen in ischaemic stroke due to embolic thrombi arising from the atherosclerotic plaques of the carotid artery and the aortic arch. The disruption of perfusion would result in definite tissue death within the cerebral parenchyma, while neuronal and glial injury develops within the penumbra, as ischaemia progresses after vessel occlusion.[21,22]

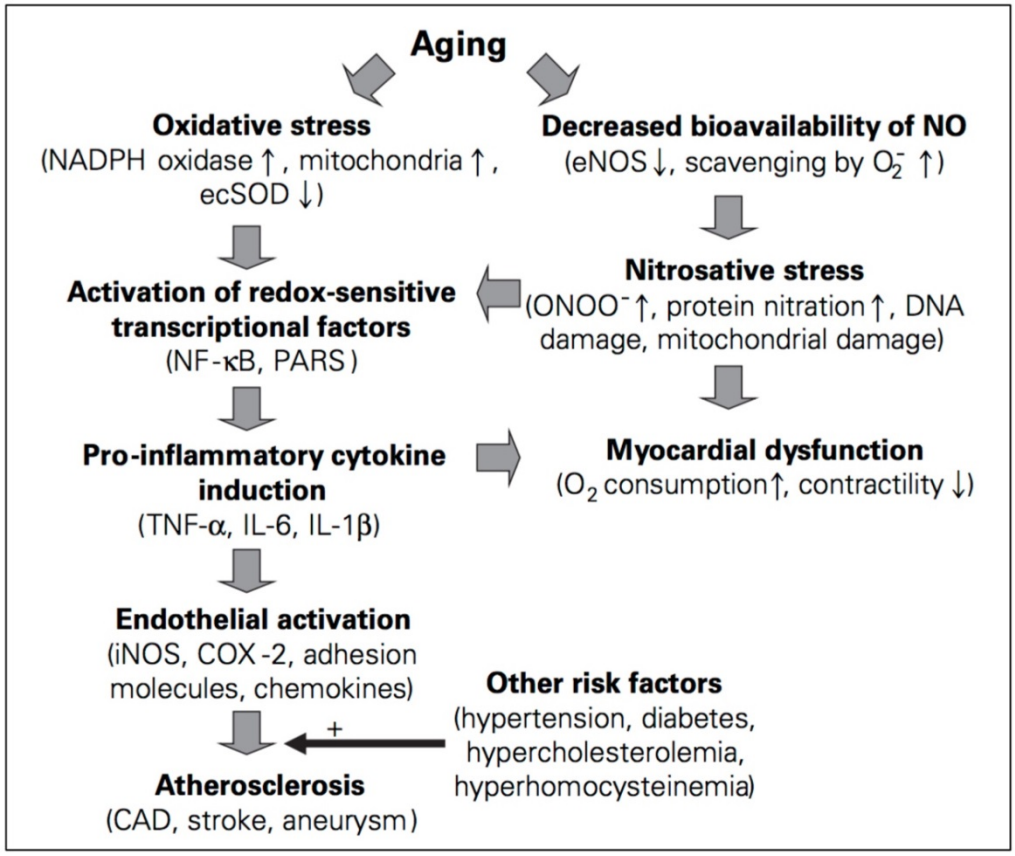

Figure 1.4.1 Diagram illustrating ageing-induced vascular inflammation. Adapted from Ungvari et al.[19]

However, the onset of neurodegeneration is not only confined to such dramatic events. Instead, it is also present in a chronic setting, underlined by transient cerebral hypoperfusion, quiet ischaemia and cerebral hypoxia, due to cerebral small vessel disease (SVD).[21,22] Such outcomes now contribute to neurological disease in increasing prevalence, with well-defined disruptions to the microcirculation of the deep subcortical brain. Pathology within these small vessels are crucial in several neuromotor conditions, distinct from the traditional parkinsonian hypotheses.[23] Vascular parkinsonism (VaP) is a prime example of this. The condition is part of a spectrum of parkinsonian diseases, yet remains distinct in its presentation.[24,25,26]

1.5 Parkinsonisms

The term "Parkinsonism" defines a spectrum of conditions that are clinically characterised by the presence of tremor, rigidity, gait impairment, altered posture and bradykinesia.[27-29] Although the diagnostic accuracy of clinical assessment and neuroimaging can be as high as 85% in cases of idiopathic Parkinson's Disease (iPD), a considerable overlap exists between conditions.[23,24]

Neuropathological examination of post-mortem brain tissue remains the superior method in reaching a conclusive diagnosis, assessing the stage and severity of the condition and the existence of co-existing pathologies.[30] Neuropathological assessment relies on the identification of misfolded proteins including phosphorylated tau and α-synuclein, often in association with deposits of β-amyloid, TDP-43 and p62. The morphology of protein aggregates, the cell types affected and the site and extent of deposit accumulation are also considered.[23,31]

iPD, Lewy Body Dementia (LBD) and Multiple System Atrophy (MSA), are characterized by abnormal accumulations of α-synuclein and are collectively termed synucleinopathies. Progressive Supranuclear Palsy (PSP) and Corticobasal Degeneration (CBD) are characterised by abnormal accumulations of phosphorylated tau and are termed tauopathies.[23] Vascular Parkinsonism (VaP) is part of the spectrum of parkinsonian disorders, but unlike its allied conditions, it is characterised by the presence of cerebrovascular disease, a prominent feature of the ageing brain.[24]

1.6 Vascular Parkinsonism (VaP)

MacDonald Critchley first described VaP as "Arteriosclerotic Parkinsonism" in 1929, suggesting the link between cerebrovascular disease and the signs and symptoms of parkinsonian disease. The hypothesis was scrutinised due to a lack of neuropathological evidence, causing the validity of

"Arteriosclerotic Parkinsonism" as a distinct clinical entity to be questioned.[27,28,31,32] FitzGerald and Jankovic revisited the condition sixty years later as "Lower Body Parkinsonism" and highlighted the lack of upper limb neuromotor dysfunction, distinguishing it from iPD. The emergence of magnetic resonance imaging (MRI) allowed the visualisation of subcortical vascular disease and white matter changes, that were both attributed to the condition.[24,27]

It was only recently that several authors introduced the term VaP to better clarify the condition. Sibon and Tison defined VaP as parkinsonism purely following stroke, without a previous clinical diagnosis of iPD.[27] This definition was restrictive and inclusive of only a minority of patients. Patients with VaP can additionally present with dementia, sensorimotor changes and pyramidal deficits. The neuropathological diagnostic criteria for VaP was then refined to include patients with atypical parkinsonism that is caused by microscopic infarctions in the basal ganglia with ongoing subcortical SVD.[24,27,28,31]

1.6.1 Epidemiology

VaP is a disease of elderly, with 80% of patients aged over 65 years. It is most prevalent in patients aged between 66-90 years, averaging at the 8th decade of life[30,33] More men are affected than women.[31,33,34] A lack of an unifying diagnostic criteria, has resulted in a poor understanding of disease incidence and prevalence. Silveria et al. suggests that VaP accounts for at least 10% of all cases of parkinsonism.[26]

Two large studies from Spain and Italy, each suggest a disease prevalence of 4.4% and 12% respectively.[35,36] Data from the Parkinson's UK Brain Bank estimated that 1 out of 8 cases of VaP was misdiagnosed as iPD during the patient's lifetime. Moreover, a fourth of patients who were clinically diagnosed with iPD had cerebrovascular changes that could have indicated

an alternative diagnosis.[31,37] It is also estimated that nearly 30% of stroke patients, exhibit parkinsonian signs. The actual prevalence and incidence of VaP is certainly higher than previously estimated.[31] The condition will increase in frequency as the population ages.

The presence of hypertension in most cases of VaP compared to iPD suggests that cardiovascular and cerebrovascular risk factors should be considered in the condition.[31] Other relevant risk factors include diabetes, hypercholesterolaemia, smoking, a family history of arteriopathy and obesity, but these have not been studied extensively.[24,26,31,38] Zijilmans et al. postulated that cerebrovascular disease can be a sole cause of VaP without the influence of other lesions. This observation indicates a neurodegenerative component in VaP pathogenesis.[39] Sibon et al. also clarified that any stroke affecting the nigrostriatal pathway could cause VaP. However, the incidence of VaP in subjects with basal ganglia infarctions are very low at only 9%. This value is higher in stroke affecting the bilateral lentiform nuclei, and the highest in brains with lacunar infarctions in the presence of white matter lesions (WML). 36% of patients with VaP experience lacunar strokes in the brain.[27,40,41]

1.6.2 Clinical Features

Disturbance in motor function associated with VaP occurs bilaterally and symmetrically, affecting the lower limbs resulting in a shuffling gait, altered posture, poor balance and an increased likelihood of falls. Almost 50% of patients face significant morbidity from falls. The National Institute for Health and Care Excellence (NICE) estimates that 30% of people older than 65 years fall at least once yearly. This statistic will be greater in VaP, costing a health and social care expenditure of more than £2.3b annually.[31,39,42] Unlike iPD, a resting tremor is not a feature of VaP. However, cases with upper limb motor dysfunction have previously been considered as VaP. Additional characteristics such as dementia, speech difficulties, pyramidal

deficits, sphincter function disturbances and sensorimotor changes are concomitant with the condition. Cognitive decline is rapid in most patients, presenting halfway through the duration of disease. Patients with VaP often face disability and institutionalised care, resulting in a poor quality of elderly life.[26,27,28,31]

The clinical identification and diagnosis of VaP is challenging and the lack of a unifying disease criteria poses a negative impact to diagnostic precision.[30,43] Dugger et al. notes that the diagnostic accuracy for parkinsonian conditions is less than 100%, and a second working diagnosis must be considered when planning long term management for patients.[23,39,44]

Novel diagnostic approaches have been proposed since 2004. A neuroimaging study clarified the use of T1-weighted MRI scans to anatomically differentiate VaP, PSP and iPD. The study found that midbrain atrophy was a likely feature in VaP and PSP compared to iPD. Meanwhile, differences in surface area ratios of the midbrain to the pons allowed the discrimination of VaP from PSP. Atrophy of the midbrain was more commonly related to cerebrovascular disease than pons atrophy. Meanwhile, Katzenschlager et al. proposed testing olfaction as a means to discriminate VaP from iPD.[45,46]

The validity of VaP as a unique clinical condition is without doubt, but accurate diagnosis of the condition remains a challenging subject. The gold-standard remains correlating clinico-neuropathological opinion.

1.6.3 Treatment

The rationale for VaP treatment initially followed that of iPD. Dopaminergic medications such as L-dopa are only effective in a minority of patients with VaP. The strategies for treatment have not yielded significant success. Several studies investigated novel approaches to explore new treatments for the condition.[26,27,28,31]

The role of subthalamic nucleus stimulation and transcranial magnetic stimulation have been proposed but the evidence is too preliminary for clinical applications.[47,48] L-threo-dops, a noradrenaline based medication had previous success in some trials, but the result of this has not been widely accepted.[31,49] Ondo et al. explored a more invasive approach. Patients with VaP have a common neurological feature with Normal Pressure Hydrocephalus (NPH). Enlarged ventricles within the brain, in both conditions suggested a potential overload of cerebrospinal fluid (CSF), which can be resolved by removing the excess fluid. 35-40mls of CSF was removed from 40 patients with VaP. Nearly 40% of the study subjects reported improvements in gait and motor function after lumber puncture. Though this study has generated positive results, the risk-benefit of repeat lumbar punctures or the insertion of a permanent ventricular shunt remains to be investigated.[28,31,50]

Since VaP has a strong link to cerebrovascular disease, a similar profile of risk factors does apply to the condition when considering cardiovascular disease. Treatment and prevention must address the common determinants of both conditions. These include, hypertension, diabetes, smoking habits, obesity, the use of anticoagulation medicines, and hypercholesterolaemia. Though no large studies have validated the impact of these factors on VaP, first principle suggests the control and treatment of these factors.[31]

Currently, best treatment and care is available via a holistic multidisciplinary approach. It is important to ensure that patients remain active and functional while dealing with progressive decline in physical function. Additional input from palliative care is essential when VaP reaches an end of life scenario. This remains best treatment until further understanding of VaP disease mechanism, is elucidated. Otherwise, there remains no cure, nor effective management for this condition.

1.7 Anatomical Substrate and VaP Neuropathology

For coordinated and refined movements, the primary and accessory cortices require control from the basal ganglia. These subcortical structures are present, bilaterally, deep within each cerebral hemisphere.[29,51,52] The basal ganglia are groups of autonomous neurones which exert control on motor function while governing several other roles in cognition, behavior and emotion. The basal ganglia are primarily compromised of the caudate nucleus, putamen and the globus pallidus, while the subthalamic nucleus, the substania nigra (SN) and the pedunculopalatine nucleus form a network of associated nuclei.

The basal ganglia exert control over motor function via excitatory and inhibitory signals transmitted through a complex set of multiple efferent and afferent loops, forming several control networks with re-entry projections.[52] The nigrostriatal pathway is the primary mechanism, for motor control in the basal ganglia. Dopamine is the key neurotransmitter, which enables the connection between the dorsal striatum and SN.[53] Dopaminergic neurones allow fine motor control through the direct and indirect, D1 and D2 pathways.[29,53]

As described previously, damage can be sustained from ischaemia, hypoxia, trauma, the deposition of misfolded proteins and the pathological processes associated with ageing. Cerebrovascular disease is a likely mediator of damage.[30,31] Thanvi et al. classified three pathological changes in the brain that related to the presentation of VaP.[30] The first is lacunar state, which is characterised by numerous small, smooth walled cavities called lacunes in the brain. The condition is a result of small strokes due to hypertension and atherosclerosis. This can also lead to cognitive and psychological deterioration.[54]

The second is chronic SVD, which presents as periventricular or subcortical WMLs.[31,38] These are associated with vascular risk factors.[28] Furthermore, Chen et al. associated severe WMLs with worsening VaP.[38] It is likely that

WMLs are due to ischaemic and hypoxic changes which result in inflammatory responses in the basal ganglia.[28] A close correlation exists between WMLs, lacunar infarctions and SVD in VaP, suggesting a possible hallmark of the condition.[55] Literature showed that tissue perfusion in the striatum was reduced in patients with gait disturbances.[56] Patients who experiences these changes suffer from intermittent periods of ischaemia and hypoxia due to poorer tissue perfusion in the basal ganglia. De Reuck showed that the presence of WMLs and striatal ischaemia, is a result of SVD.[55,57] Vascular damage within diseased vessels results in inflammatory responses that lead to the chronic damage of neurons and glia.[28,30] Striatal ischaemia also results in damage to its distal connections. This is best seen through the loss dopaminergic neurones in the SN after focal inflammation due to hypoxia in the dorsal striatum proximally.[31,58]

Thanvi's final pathological classification refers to infarction. The occurrence of haemorrhagic and/or ischaemic strokes within the basal ganglia, that would result in parkinsonian symptoms secondary to the area of damage and neuronal death.[31]

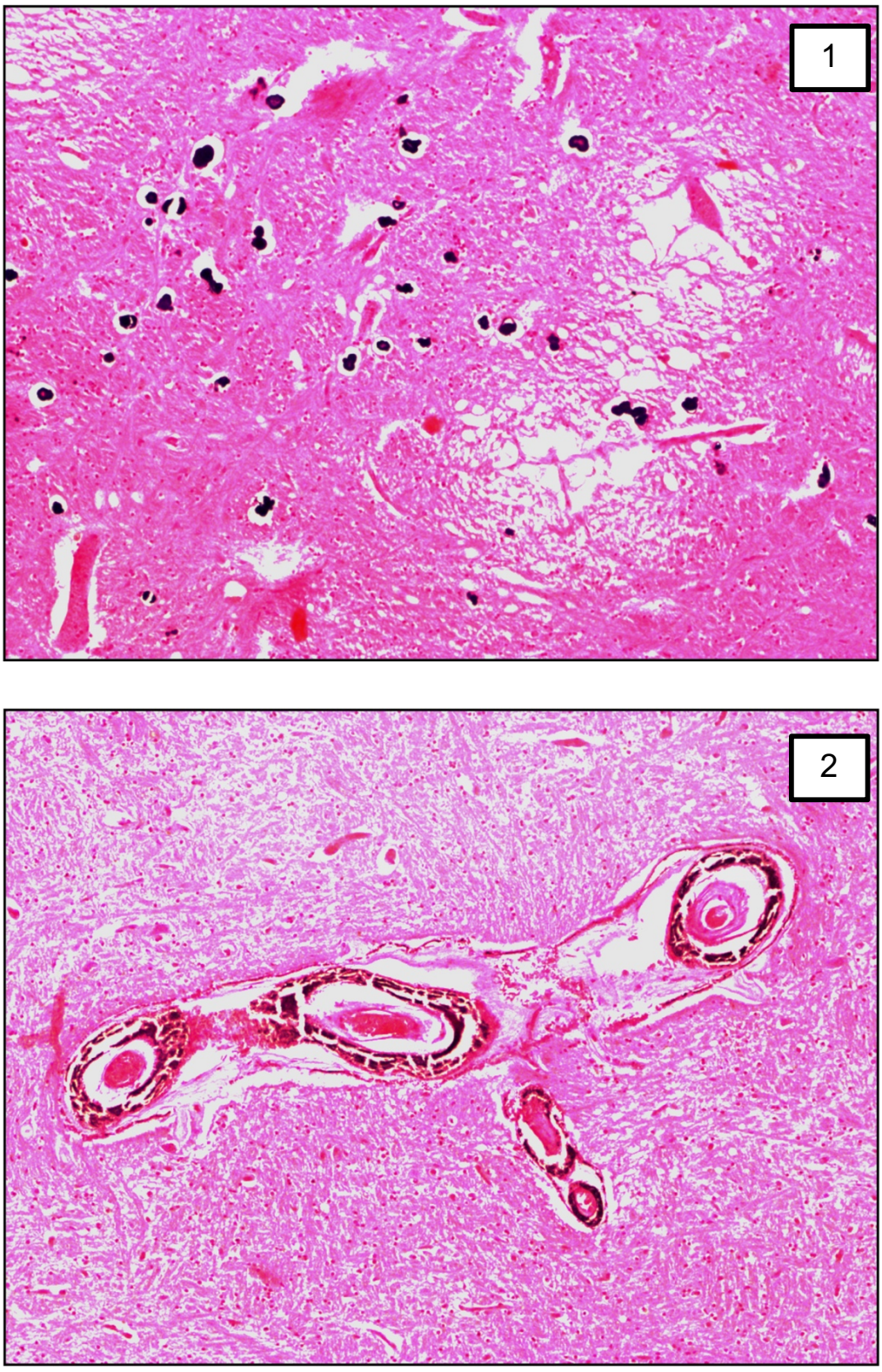

Figure 1.7.1. Fibrosis, thickening and calcification of; (1) small blood vessels in the external pallidus due to SVD, (2) medium sized blood vessels in the internal pallidus due to SVD. The vessel lumen in narrowed, suggesting a decrease in normal perfusion. (H&E x20)

1.8 Neuroinflammation

Neuroinflammation refers to the inflammatory responses of the CNS. Similar to inflammation in non-CNS tissue, the inflammatory processes in the brain can be triggered by a variety of factors, not limited to trauma, infection, immune dysfunction and the pathological processes in ageing.[59] The CNS is usually protected from peripheral inflammatory changes by the blood brain barrier (BBB). Endothelial dysfunction and transient low level inflammation due to ageing increases the permeability of the barrier, resulting in CNS vulnerability to peripheral inflammation. A compromised BBB predisposes individuals to inflammation in the brain.[60]

Though, inflammatory responses are naturally protective, the onset of neuroinflammation is regarded as a significant mediator for neurodegenerative diseases. The extent of this is not fully known, but the activation of resident CNS immune cells known as microglia and the migration of peripheral leucocytes into the brain are definite contributors.[59,61] Microglia are characteristic in the brain, acting as resident macrophages to mount cell mediated immune defence. These cells also carry out several other roles.[59,61,62]

Microglial response is seen when brain tissue is damaged. These surveyor cells usually remove damaged neurons and support brain homeostasis without the induction of inflammatory reactions. These cells also function as antigen presentation cells (APCs) in response to infections within the CNS. This role is important as peripheral APCs and antibodies are normally unable to bypass the BBB. This ensures adequate immune response to infection in the CNS. Moreover, microglia promote repair and regrowth of neuronal connections by removing damaged dendritic branches and dysfunctional synapses. This allows for renewed synaptic development with better integrated neuronal connections.[63]

In iPD, the loss of dopaminergic neurones correlates to a local pro-inflammatory state where counteracting anti-inflammatory responses are less adequate.[61] The use of the anti-inflammatory drug ibuprofen is hence notable. Ibuprofen has been documented to reduce the risk of developing the iPD, but similar findings were not observed with other NSAIDs. This is due to neuroprotective effects specific to ibuprofen.[64] Lehmann et al. clarified that ibuprofen was peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, which could explain this specific neuroprotection.[65] However, the prospect of other anti-inflammatory treatments for neurological diseases is promising.

1.9 The Pre-clinical Mouse Model

The link between ischemia and hypoxia associated with SVD, neuroinflammation and the loss of dopaminergic neurones was proposed by our colleagues, Roriguez-Grande et al. The study proposed a mouse model which studied the onset of ischaemia in the basal ganglia in response to occlusion of the middle cerebral artery. This induced ischaemia within the dorsal striatum which was succeeded by the onset of neuroinflammation and subsequent neuronal loss distally in the SN. Early inflammatory action is closely related to the potential loss of dopaminergic neurones in the nigrostriatal pathway. The model provided a potential explanation for neuromotor impairment in parkinsonian disease, mediated by striatal ischaemia. These findings were applicable to the SVD mediated changes seen in the basal ganglia of elderly patients with movement disorder.[26,27,28,31,58]

2.0 Aims

The aim of our work was as follows:

- To determine the neuropathological substrate of VaP
- To evaluate the differences between VaP and iPD with comparison to age-matched controls with markers for neuroinflammation, dopaminergic neurones and synaptic density.
- To validate the mechanism of disease in VaP, as proposed by a previous pre-clinical mouse model, involving the chronic and transient state of cerebral hypoperfusion.
- To demonstrate VaP as a distinct condition of the elderly.

3.0 Methodology

The clinico-pathological features of 819 brains with movement disorders available at the Parkinson's UK Brain Bank at Imperial College, London were reviewed. We selected 11 cases of VaP, 4 of early stage iPD (LBPD Braak III), 4 of late stage iPD (HBPD Braak VI) and 4 age-matched negative controls with mild SVD. Additionally, 5 control cases with severe SVD in the absence of movement disorders were selected from the Manchester Brain Bank, Manchester. Detailed clinical history and neuropathological evaluation of tissue was available for all cases.

Immunohistochemistry was performed according to standard protocol using the following antibodies: Iba1 for microglia and macrophages, GFAP for astroglia, Tyrosine Hydroxylase (TH) for dopaminergic neurons, Synaptic Vesicle 2A (SV2A) for overall synaptic density and the NLRP3 inflammasome (ASC). Immunodetection was achieved with HRP immunoperoxidase, with DAB as a chromogen and haematoxylin as counterstain.

Qualitative and quantitative analysis was carried out with ImageJ (https://fiji.sc) by examining images of the putamen, external pallidus and internal pallidus obtained via brightfield microscopy. Iba-1 and GFAP immunostains were quantified by direct cell counting, while TH and SV2A were measured with digital optical density (OD). T-tests were performed for statistical analysis.

4.0 Results

The average microglial density was 10 times higher in VaP compared to control tissue. A 2-fold increase in microglial density was seen in VaP compared to iPD (external pallidus; $p=0.0088 < 0.05$, internal pallidus; $p=0.0168 < 0.05$)

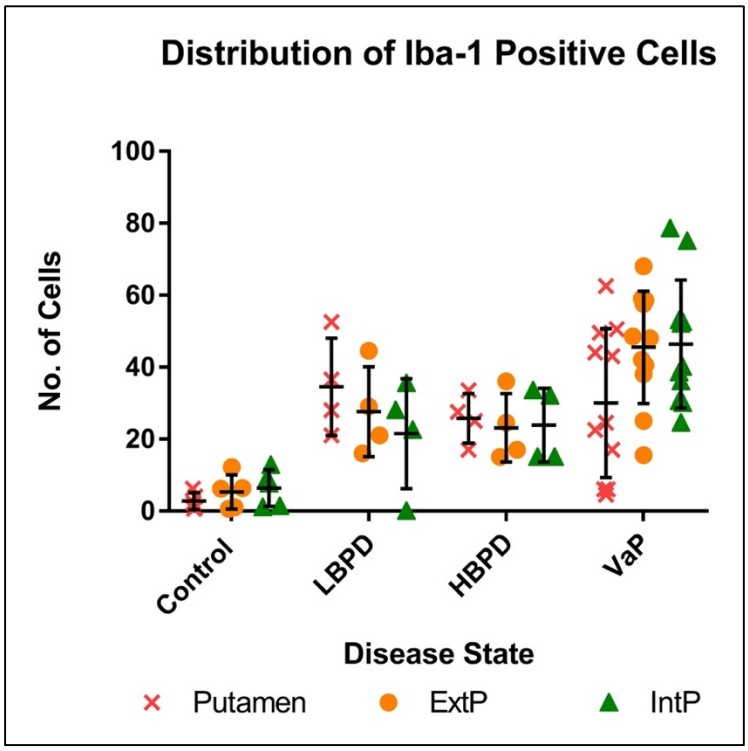

Table 4.0.1. Dot plot representing mean Iba1+ cell counts in iPD and VaP disease states against aged matched controls.

Microglia showed morphology of activation in VaP compared to thin and ramified cells seen in iPD and control tissue. This signified an activated inflammatory state. Activated microglia were also more commonly ASC positive indicating greater NLRP3 inflammasome activation in VaP. This suggested the downstream upregulation of IL- 1β.

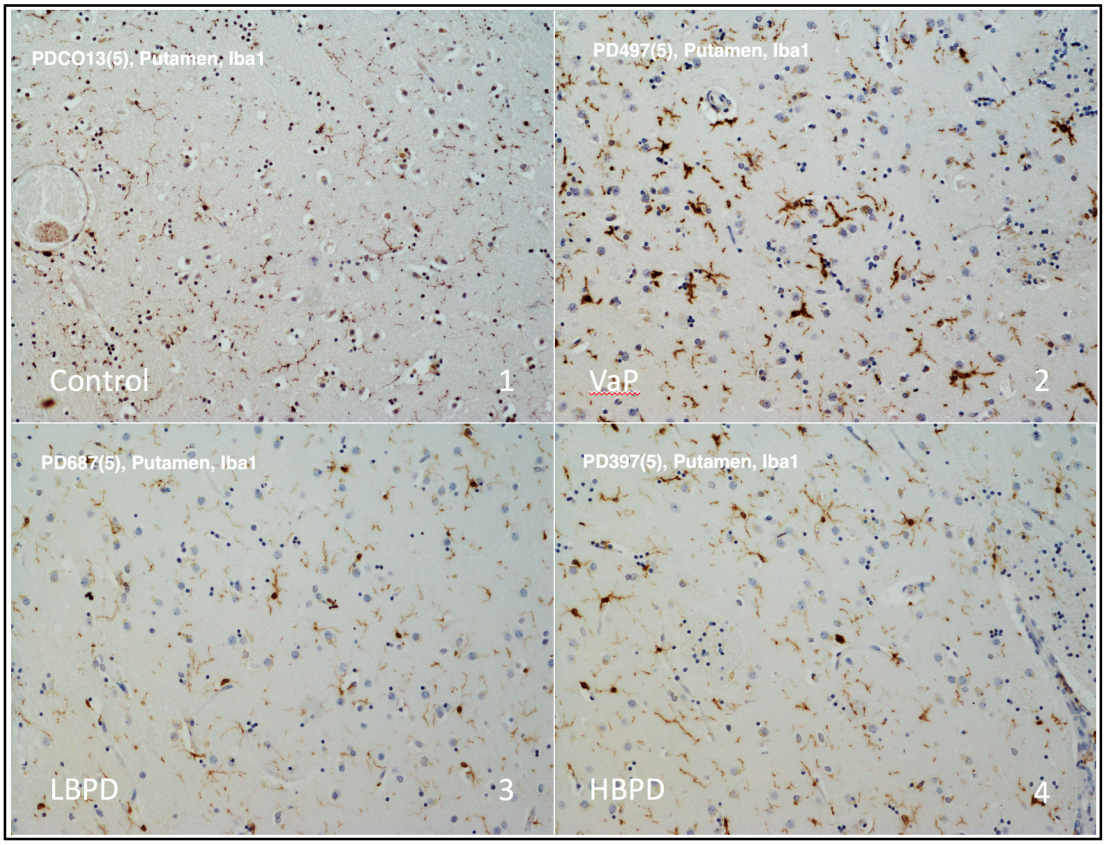

Image 4.0.2. Comparison of Iba1+ immunostaining displaying microglia morphology in various disease states and control tissue. The appearance of microglia with less projections and a greater cytoplasmic focus on the cell body is seen in image 2. (DAB-H x20)

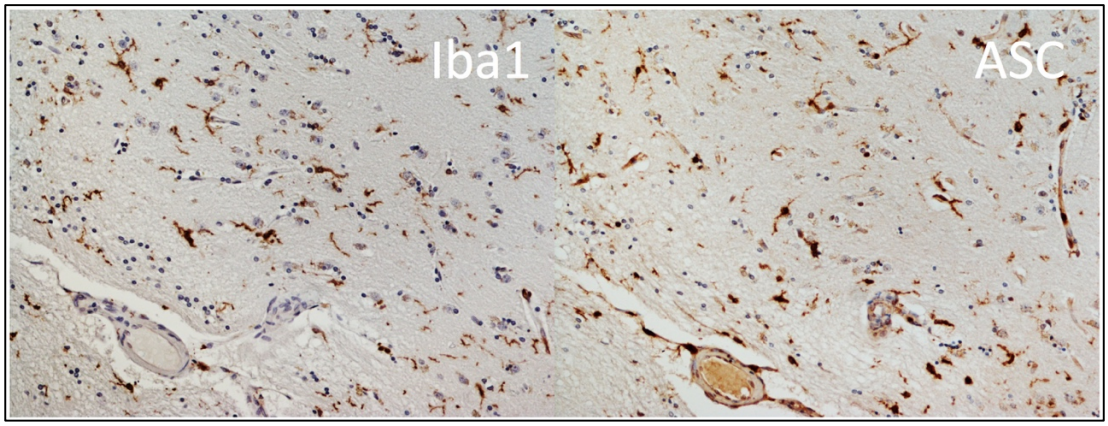

Image 4.0.3. Basal ganglia displaying co-localisation of Iba1+ and ASC+ cell types, denoting positive inflammasome activation in microglia in VaP. (DAB-H x20)

Astroglial density was similar in VaP and late stage iPD with no significant differences observed in the basal ganglia. However, a prominent reactive astrocytosis was seen in early stage iPD, with significant differences seen in the basal ganglia. (putamen; p=0.0026 <0.05, external pallidus; p=0.0034 <0.05, internal pallidus; p=0.0156 <0.05).

The loss of dopaminergic terminals in the putamen was widespread in all conditions compared to control tissue. A ten-fold loss in dopaminergic neurones was significant in the putamen (p=0.0428, <0.05) and internal pallidus (p=0.0280, <0.05) in VaP compared to controls. Dopaminergic loss was similar in both stages of iPD in the dorsal striatum.

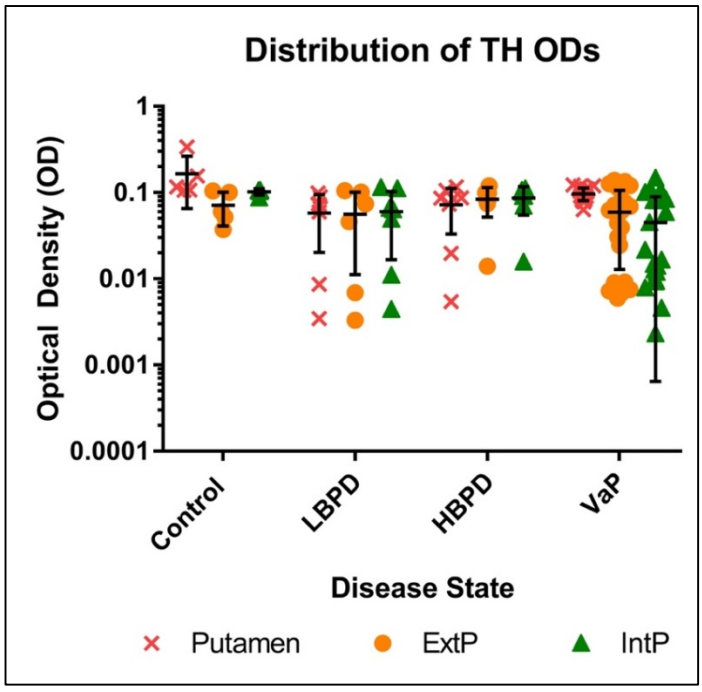

Table 4.0.4. Dot plot representing mean optical densities (OD) of TH+ neurons in iPD and VaP disease states against aged matched controls.

A marked decrease in synaptic density was seen in all diseases compared to control tissue. A two-fold loss was seen in all three regions in VaP and late stage iPD with no significant differences observed. However, similar losses are only seen in the putamen in early stage iPD. This potentially suggests the putamen as the first area affected in the condition.

When comparing our results with SVD controls, similar immunostaining was observed for astroglia, dopaminergic neurones and synaptic density. However, differences were seen in Iba1 and ASC immunostaining. Cells in this group were non-activated, significantly reduced and less commonly ASC positive. It is unlikely that this group exhibited similar neuroinflammatory features as seen in the VaP and iPD groups.

5.0 Discussion

In this study, we have investigated the anatomical substrate of VaP and compared respectective pathological changes in the basal ganglia to brains with iPD and aged matched controls. Our results documented for the first time a significant increase in a neuroinflammatory response in the basal ganglia of subjects with VaP compared to iPD and age-matched controls. In addition, we proved that the basal ganglia of subjects with VaP shows severe loss of dopaminergic neurons similar to iPD

5.1 Novel Findings and Improved Understanding

No studies have previously investigated in detail the histopathological changes of VaP. The pathogenesis of VaP has long been debated since its proposal nearly 90 years ago due to the lack of firm neuropathological evidence supporting the link between VaP and cerebrovascular disease. Neuroimaging studies showed a correlation between the clinical presentation and vascular changes in the basal ganglia but such findings do not always correlate with histological evidence of SVD. The results presented here are novel, and contribute to the understanding of disease mechanism in VaP, while providing a potential avenue for future research in neurogeriatric medicine.[26,27,28,31,66]

The strength of this study first lies in the access of post-mortem brain tissue samples of 11 subjects with VaP that are both clinically and neuropathologically fully characterised. The brains were supplied by the Imperial College Parkinson's UK Tissue Bank and carefully selected from 819 brains with a diagnosis of parkinsonism and 120 control brains. Criteria of selection was strict to avoid co-morbidities such as sepsis that could have contributed to increasing neuroinflammation in the tissue examined, and severe cerebrovascular pathology such haemorrhagic or ischaemic stroke.

Finally, we used macrosections of the basal ganglia to allow for an extensive evaluation of the regional anatomy and avoid bias of sampling.

The nigrostriatal pathway is a two-way connection between the SN and dorsal striatum, mediated by dopaminergic neurones that function as modulators of gross movement. Disruption of the three components, the dorsal striatum, the intermediate nigrostriatal connection and the substantia nigra is known to be the underlying cause of parkinsonian syndromes but the exact geographical dysfunction of neurotransmission has never been anatomically proven in VaP.[29,53]

The view on neuroinflammation in VaP was previously uncertain as its presence can be due to either a primary, secondary or tertiary cause, each with decreasing intensity. It was unlikely that neuroinflammation in VaP primarily originated from a true cause of immune dysfunction within the brain, as seen in multiple sclerosis. It was more likely that the neuroinflammatory presence in VaP was secondary to chronic and transient hypoperfusion due to extensive small vessel disease resulting in hypoxia in the extremely sensitive regions of the basal ganglia. It was also likely that the extent of neuroinflammation in VaP is a tertiary accumulation of ongoing and chronic baseline CNS inflammation due to the gradual and overwhelming pro-inflammatory status seen in ageing, coupled with a chronic deterioration of the BBB. No previous studies have clarified the extent and character of neuroinflammation in VaP.[28,31,58,67-70]

5.2 VaP Neuropathology and Practical Implications

It is true that cerebral SVD can result in parkinsonism. Both SVD and VaP are closely related and have previously been defined as distinct clinical entities by published criteria. The presence of parkinsonism with SVD in the presence of a normal appearing SN is definitive of VaP. Kalaria et al. highlighted the six distinct subtypes of cerebrovascular disease of which

subtype II is of particular interest. The proposed Newcastle categorisation classified subtype II as cerebrovascular associated with microinfarctions.[66,71]

The category required more than three microinfarctions with a minimum diameter of 5.0mm, the presence of SVD, hyalinisation, chronic amyloid angiopathy, lacunar infarctions and perivascular changes in greater than three coronal levels. The age of these vascular lesions should correspond with the elapsed time since the disease began, taking the appearance of subtype II vascular pathology as a marker for disease manifestation. Several conditions are related to this subtype. Patients post-stroke, cases with extensive white matter lesions, atherosclerosis, collagen or amyloid angiopathy, lipohyalinosis, hypertensive vessel disease and vasculitis can manifest with the vascular changes mentioned above. Almost all of these conditions are present in tissue donors with VaP and in ageing brains. Subtype II CVD is a hallmark of VaP pathology in the brain.[66,71]

The clinical criteria for VaP is less definitive with diagnosis most confidently given to patients with parkinsonism from a substantial cerebrovascular cause. This is usually reserved for patients with parkinsonism as a result of stroke.[31] However, this definition is narrow, given the various signs and symptoms associated with the condition, that present without the onset of acute cerebral ischaemia. VaP is hence accepted as bilateral and symmetrical functional decline in the lower limbs which results in postural instability, poor balance and an abnormal shuffling gait.[27,28,31,39] Though the presence of an upper limb resting tremor is atypical, a diagnosis of VaP has been extended to this and several other symptoms such as dementia, speech difficulties, sensorimotor changes, pyramidal disturbances and decline in sphincter function.

Loss of dopaminergic terminals in VaP explains motor dysfunction. However, the underlying mechanism of disease is different to iPD. Though the exact molecular changes resulting in cell death in response to chronic hypoperfusion is elusive, SVD remains a consistent hallmark of VaP.

Therefore, the impact of cardiovascular risk factors should be considered as avenues for clinical intervention in VaP prevention.[31,39] Our findings support the role of SVD as the key contributor to late-life motor impairment. This hypothesis further explains the functional decline of patients with vascular dementia; a disease with similar cerebrovascular aetiology. Deterioration in these patients is not only due to decline in cognition but more likely due to motor impairment, contributing to elderly disability.[72]

Clinical diagnosis alone is not specific as there is a lack of empirical evidence that relates to neuromotor decline in the brain. MRI and PET neuroimaging with brain electrophysiology may add predictive value for VaP, but the lack of neuropathological evidence is a significant gap in understanding the condition. We have shed some light on this matter.[31,39,73]

5.3 Neuroinflammation in VaP

The primary aim of this project was to demonstrate the neuroinflammatory component of VaP. This study is first to demonstrate significant signs of neuroinflammation in human brains diagnosed with VaP. The high numbers of microglia seen presents a novel case for ASC-mediated IL-1β upregulation in disease development. The results display a prominent microglial influence in VaP pathogenesis, suggesting a strong link between the presence of SVD induced hypoperfusion and hypoxia, with a peripheral ageing-induced pro-inflammatory state, to a chronic inflammatory response in the basal ganglia.

The hypothesis of this study comes from findings of a pre-clinical mouse model that suggested neurodegenerative changes in response to hypoperfusion. The model suggested the distal and delayed loss of neurones in the SN after inducing ischaemia in the dorsal striatum. The loss of substance P precedes the onset of neuroinflammation, concluding in neuronal death within the SN. The model provided a brief insight into the possible outcomes of striatal hypoxia due to occlusion of the middle cerebral

artery. The high sensitivity of the deep brain structures is due to the anatomical distribution of the supplying vessels. The basal ganglia are primarily supplied by the deep perforating lenticulostriate vessels which branch from the middle cerebral artery. The induction of vessel occlusion was a valid starting point in inferring similar circumstances in VaP due to SVD. However, the model does not replicate the hypoxic and hypoperfusive conditions experienced by the human basal ganglia at a transient state throughout years of the patients' lifetime. The extent of poor basal ganglia perfusion in the mouse model, does not mirror the actual disease process in human patients.[58]

However, the model is nonetheless important as the changes to the middle circulation of the brain may exist in a continuum, yielding an early speculation of similar changes in VaP. The obvious difference is that the SN is maintained in VaP while the pre-clinical model suggested focal neuronal loss. The effect of hypoperfusion time against SN health remains to be evaluated. The extent of hypoperfusion-induced changes in the mouse basal ganglia may in fact be representative of VaP, given that the neuroinflammatory response is dependent on a similar degree of vascular dysfunction *in-vivo*. This suggests a lasting neuroinflammatory presence in the basal ganglia in a chronic and transient state of hypoperfusion, while the health of the SN is conversely maintained. However, this hypothesis is speculative.

The uncontrolled presence of neuroinflammation induces oxidative stress within susceptible neuronal populations leading to neurodegenerative changes. Microglia release a variety of pro-inflammatory substances when subject to changes in the microenvironment that lead to a prolonged disruption in normal homeostasis.[74,75,76] Among cytokines, chemokines, reactive oxygen species and prostaglandins, the chief mediator of said responses is the cytokine IL-1β.[77] Naturally, the characterisation of neuroinflammation should have revolved around the detection of this marker along with TNF-α, COX, iNOS and IL-6, which are well studied

members of the inflammatory cascade. However, the use of formalin fixed, paraffin embedded tissue with post-mortem delay would have rendered the immunodetection of these markers futile. The true extent of inflammatory expression would have been a gross underestimation of its true extent in VaP. This is due to the labile nature of the microglial cytokines, hence the NLRP3 was considered as a better candidate.

The NLRP3 inflammasome, is a major multimeric protein complex formed by the NLRP3, ASC and Pro-caspase-1 proteins. The structure acts as a catalyst for the release of caspase-1, which subsequently upregulates the expression of IL-1β from its pro-inflammatory precursor state. The presence of the NLRP3 inflammsome is innate in microglia, responding to pathogens via pathogen-associated molecular patterns (PAMPs) and stressors from the microenvironment via danger-associated molecular patterns (DAMPs).[78]

Immunostaining was preferred for ASC due to the stable nature of this protein compared to the volatile cytokines. The neuroinflammatory presence in paraffin embedded tissue was best demonstrated with this component of the NLRP3 inflammasome. The widespread positive immunostaining for ASC inferred a downstream IL-1β mediated inflammatory state most prominent in VaP followed by late and early stage iPD. This study was first to clarify a positive expression of the NLRP3 inflammasome in VaP, in the absence of α-synuclein. Sole evidence from Codolo et al. suggested that the downstream expression of IL-1β was dependent upon monocyte NLRP3 activation by α-synuclein in iPD.[79] ASC presence in the microglial cytoplasm infers the induction of the NLRP3 inflammasome regardless of a-synuclein occurence. It is not known whether there exists an inflammasome activator specific to VaP, but this is an unlikely occurrence.[78,79]

5.4 Future Work

The results of this study should be complimented with further studies.

The exact localisation of ASC within microglia and astroglia can be determined with the use of double immunofluorescence and visualisation with confocal microscopy. This is of particular interest as the location of ASC either within the nucleus of cytoplasm infers changes in the NLRP3 inflammasome activation and downstream expression IL-1β activation.[78] The relationship between ASC location within microglia, microglial density in tissue and cellular morphology should be investigated in parallel. A link between microglial change from resting to an amoeboid state parallel to the movement of ASC from the cellular nucleus to the cytosol would clarify the relationship between microglial activation and the action of the NLRP3 inflammasome within the central nervous system.[74,77,78,80,81]

To support the results obtained with immunohistochemistry, we should quantify the transcripts of the cytokines IL-1β, IL-6, TNF-α, substance P and enzymes such as iNOS and COX using quantitative polymerase chain reaction (qPCR). Protein quantification with western blotting would also be helpful to validate the results of synaptic and TH density. The exact cytotoxic mechanism of VaP remains to be investigated, and the exact role of infiltrating leucocytes in response to a damaged BBB and a pro-inflammatory state is elusive.

The dopaminergic connections of the nigrostriatal pathway are certainly central to the development of parkinsonian signs. However, the role of neuronal projections from the cortex and thalamus to the striatum must be investigated as well. These connections utilise GABA as a primary neurotransmitter for inhibitory actions. These neurones, known as medium spiny neurones (MSNs) are a significant component of the striatum and basal ganglia.[82,83] These D1-type and D2-type cell phenotypes are significantly involved in both the direct and indirect pathways of fine motor control within the dorsal striatum. Furthermore, the role of dorsal striatal cholinergic

interneurons must be considered as well. The dysfunction of both these neurotransmitters, their carrier neurones and respective synapses can contribute to neuromotor disease.[84,85,86]

Though a histological biomarker is suggested, the use of IHC for VaP diagnosis in brain tissue is of less importance in clinical practice, as diagnosis should aim to benefit the living patient. The translation of findings from this study, into a neuroimaging biomarker is of great potential for both disease detection and monitoring. Literature clarifies several studies that have used radioligand markers for Dopamine Transporter (DAT), mitochondrial translocator protein 18kDa (TSPO) and SV2A for the measurement of microglial mediated neuroinflammation, dopamine expression and synaptic density in living subjects.[73,81,87-89] The combined used of these ligands for the determination of disease progression in a cohort of susceptible patients with cerebrovascular disease and subcortical hypoperfusion should be explored. This would allow the diagnosis of VaP, the detection of potential disease and risk stratification for susceptible elderly patients. This would complement the rationale of pre-emptive pharmacological interventions with dopaminergic and anti-inflammatory medications in early stage disease as treatment and perhaps prevention prophylaxis. Recent evidence suggests the use of mefenamic acid as a potential NLRP3 inflammasome inhibitor. Meanwhile, early therapeutic interventions with medications such as minocycline that influence the cytotoxic activity of microglia could be explored.[90,91]

6.0 Conclusion and Impact

We think our results have led to a clearer understanding of the pathogenesis of VaP and are relevant enough to; refine diagnostic criteria, identify preventive measures to slow the condition and plan treatment for the elderly. The significant increase in microglia in VaP suggests neuroinflammation is a key mediator of the condition. The expression of ASC in activated microglia suggests the downstream upregulation of IL- 1β. The combination of increased activated microglia, loss of synaptic density in the putamen and internal pallidus along with a 10-fold loss of dopaminergic neurons is unique to VaP. These exact changes have never been studied in human tissue and can be considered a potential biomarker of disease.[92,93]

Dopaminergic neurons and neuroinflammation can be investigated in vivo using PET imaging with radiotracers that bind dopamine transporters[88-89] in neurons of the basal ganglia or tracers that bind molecules expressed by activated microglia.[81] Although the sequence of events that cause damage of dopamine terminals are difficult to establish, the possibility that microglia are the mediators of synaptic degeneration may suggest the use of molecular imaging for early identification of basal ganglia damage and early therapeutic interventions with anti-inflammatory and microglia modifying medications. Previous studies attempted treatment of VaP with dopamine agonist such as L-Dopa but results were discouraging. Our results suggest that the lack of efficacy of L-Dopa could have been due to damage of the nigrostriatal pathway that was too severe and already irreversible. A therapeutic window may exist, once identified, could potentially reduce the severe morbidity faced by elderly patients due to subcortical SVD.[26-28,31,34,39]

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