Pathology of the spinal cord in progressive multiple sclerosis (primary progressive vs secondary progressive)

A PhD thesis submitted to the University of Nottingham for the degree of doctorate of medicine

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Abstract

Background:

Recent studies have shown that the two major forms of multiple sclerosis are different in the degree of demyelination and atrophy, degree of inflammation, and extent of axonal loss. However, the majority of the previous studies that compared primary progressive and secondary progressive multiple sclerosis were carried out at the brain level.

Material and methods:

Human post-mortem spinal cords were used to compare the two progressive subtypes. In this project, the 5 major pathological changes associated with MS were studied in the spinal cords of primary progressive and secondary progressive multiple sclerosis. These changes include degree of demyelination, atrophy of the tissue, oligodendrocytes pathology, axonal loss, and neuronal pathology.

Results:

There was significant atrophy in the spinal cords of MS compared to healthy controls, which affects mainly the upper cord levels. There is a greater degree of demyelination and atrophy affecting secondary progressive compared to primary progressive especially in the upper cord levels. Oligodendrocytes numbers are dramatically reduced in the chronic lesions of WM and GM lesions. But there was high numbers of oligodendrocytes in the normally appearing GM of secondary progressive multiple sclerosis. There was greater reduction in axonal density in the secondary progressive sample especially in the normally appearing WM. Neurons were reduced in the demyelinated grey matter regions with no difference between the two disease forms in this respect.

Conclusions:

SPMS seem to have greater degree of tissue destruction in the form of demyelination, atrophy, and axonal loss in the normally appearing WM. However, SPMS showed greater numbers of oligodendrocytes in the demyelinated areas of the WM and the GM. Although the disability scale in the two examined groups was found to be similar, the tissue damage appeared to be variable.

Abbreviations

APP	Amyloid Precursor Protein
BBB	Blood Brain Barrier
CA	Carbonic Anhydrase
CNPase	Cyclic Nucleotide Phosphodiesterase
CNS	Central Nervous System
CSA	Cross Sectional Area
CSF	Cerebro-Spinal Fluid
CST	Cortico-Spinal Tract
DH	Dorsal Horn
DMGM	Demyelinated Grey Matter
DMWM	Demyelinated White Matter
EBV	Ebstein Barr Virus
EDSS	Expanded Disability Status Scale
EP	Evoked Potentials
GFAP	Glial Fibrillry Acidic Protein
GM	Grey Matter
ICAM 1	Intracellular Adhesion Molecule 1
IF-γ	Interferon Gamma
IL	Interleukin
MAG	Myelin associated glycoprotein
MBP	Myelin Basic Protein
MS	Multiple Sclerosis
NAGM	Normally Appearing Grey Matter
NAWM	Normally Appearing White Matter
OLs	Oligodendrocytes
OPCs	Oligodendrocytes Progenitor Cells
PCP	Posterior Column Pathway
PLP	Proteo-Lipid Protein
PMGM	Partially Myelinated Grey Matter
PMWM	Partially Myelinated White Matter
PNS	Peripheral Nervous System
PPMS	Primary Progressive Multiple Sclerosis
RRMS	Relapsing Remitting Multiple Sclerosis
SPMS	Secondary Progressive Multiple Sclerosis
TGF-β	Transforming Growth Factor Beta
Th	T helper
TNF-β	Tumour Necrosis Factor Beta
VCAM 1	Vascular Adhesion Molecule 1
VEPs	Visual Evoked Potentials
VH	Ventral Horn
WM	White matter

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Chapter 1: Introduction

Multiple sclerosis (MS) is an inflammatory autoimmune demyelinating disease of the central nervous system (CNS) [1]. The disease results in multiple lesions that are characterised by loss of myelin substance as well as oligodendrocytes (OLs), which are vital elements for the proper function of the CNS. Therefore, MS can lead to wide range of symptoms.

1.1 History of Multiple Sclerosis

In the book entitled "Multiple Sclerosis: The History of a Disease" the author, Dr T J Murray, described symptoms of many patients during the 18th century who had features similar to MS [2]. The earliest described case is thought to be Saint Lidwina. Saint Lidwina was born in Schiedam of Holland on 18th April 1380. She fell on ice at the age of 15 years and broke her rib [3]. Following this, she developed weakness in both lower limbs and severe tooth pain, which are believed to have been the paraplegia and the trigeminal neuralgia of MS respectively. She also suffered from unilateral blindness and became paralysed and confined to bed. Severe bed sores complicated her condition and affected her entire body. People at that time believed that she was under the influence of an evil spirit [3]. She died in April 1433 at the age of 53 years [4]. In 1947 her remains were exhumed for analysis, and they demonstrated marks of spastic paraplegia [5].

Augustus d'Este (1794-1848) remains the most famous historic case of what is believed to have been MS. He kept detailed records of all his symptoms in his diary. Briefly, he suffered from blurred vision in 1822, which resolved spontaneously. Then he began to develop visual loss and was unable to read. He described in detail all the types of treatment he received. His condition progressed to paraplegia until he died in 1848 [5].

The German pathologist Friedrich Theodor von Frerichs (1819–95) established the first diagnosis of myelitis in 1849, which is believed to be MS. Eduard Rindfleisch (1836–1908),

was the first person to describe the perivascular cellular infiltration and neuroglial involvement as part of the disease process [4, 6].

MS as a distinct new disease was described for the first time in 1868 by the French neurologist Jean-Martin Charcot (29 November 1825-16 August 1893). Charcot and his colleague Edmé Vulpian were studying a specific type of tremor in young patients, and were trying to differentiate it from paralysis agitans caused by Parkinson's disease. Charcot and his assistant noticed that this type of tremor frequently affected young adults and was associated with paralysis. They then noticed patchy greyish plaques in the autopsy of these patients, which were scattered throughout the spinal cord and the brain. In 1866 Charcot presented three cases and differentiated MS tremor from paralysis agitans. Later, Charcot described a number of MS features, and he named it sclérose en plaque disseminée [5, 7].

Charcot also contributed largely to developing criteria for the diagnosis of MS [8]. The criteria are known as the Charcot's triad, which includes diplopia, ataxia and dysarthria. He observed these three symptoms in his housekeeper, who was affected by MS. Furthermore, Charcot was the first neurologist to provide a detailed description of MS histopathology, including loss of myelin [6, 9].

After Charcot's work there was little progress in MS and most of the research was focused on finding the disease aetiology. In 1933 the first model of MS was produced in monkeys, which was called disseminated encephalomyelitis. Then MS model was produced in mice and was called experimental allergic encephalomyelitis (EAE) [2, 4]. Until 1935 there were about 150 proposed treatment strategies for MS [4].

The world 'sclerosis', meaning hardening, was used to describe the scarring that follows the damage of the nervous system by the disease process. The disease causes demyelination

of the axons in different areas of the white matter (WM). Therefore the word 'multiple' was used to describe the disseminating characteristic of the lesions, which are disseminated in time and place. These lesions were called plaques, which are well-defined areas of demyelination and neuro-axonal degeneration. The word sclerosis had previously been used in the USA to describe another neurological disease (amyotrophic lateral sclerosis). In Britain the disease was called disseminated sclerosis. Then the name was changed to MS because of the establishment of the MS Society in the USA [10].

1.2 Epidemiology

By reviewing European hospital records and comparing them to the present MS features, Dr T J Murray was able to conclude that MS had been prevalent in the European population for many centuries, and that its prevalence did not change with time [4].

Estimates of incidence and prevalence of a disease such as MS differ between various studies. Each study investigates a different sample size and age range, and different ethnicity. Various studies may apply different criteria for the diagnosis, and thus results may vary.

Increase in the prevalence of MS is partly due to increased diagnosis, as many cases went unrecognized [11], but the latest epidemiologic studies state that the prevalence of the disease is, in fact, increasing with time [12-16].

The disease predominantly affects individuals between the ages of 20 and 40 years [16]. MS is the most common neurological disease that affects young western adults [17], and the second most common cause of neurological admissions in young adults after trauma [11]. In 2002 it was estimated that 2.5 million people are affected with MS worldwide [11]. In the UK

approximately 100,000 individuals suffer from MS, according to the MS Society in 2005, while the number reaches 350,000 patients in the USA [16].

The prevalence of MS is geographically variable according to latitude, where it reaches 60-200/100,000 in Northern Europe and North America compared to 6-20/100,000 in lower risk areas, such as Asia [16].

MS disease does not cause significant life shortening, but has a high morbidity rate and a strong impact on the social and economic status of the affected individual and the whole country. In 1995, the cost of the disease in the UK was estimated to be around £1.2 billion [18]. In a cross-sectional study, the average cost of MS was calculated in various countries for one patient for a period of three months [19]. The study revealed that the cost was variable according to the country and the stage of the disease. In the UK, according to Expanded Disability Status Scale (EDSS), a patient in stage III costs $14,622 every three months [19].

1.3 Risk factors

The cause of MS is believed to be multifactorial. The role of environmental factors in developing MS has been shown in a number of studies [15, 20], but the extent of this role is not yet certain. As genetic studies have shown genetic contribution, it is thought that MS develops in genetically susceptible individuals after exposure to triggering factors, which could be infectious factors.

1.3.1 Genetic factors

It is important to mention that geographic variation of MS epidemiology is not only dependent on environmental factors, but also depends on ethnic or genetic factors where each ethnicity usually inhabits a certain geographic area [15].

First degree relatives of MS patients have 20-50 fold higher risk of developing the disease. The most relevant gene was found on the area of the major histocompatability complex, which accounts for 10-60% of the genetic risk of MS [21]. The concordance rate in monozygotic twins is 20-30% [16, 21, 22]. Moreover, the concordance among monozygotic twins has been also affected with latitude and with the time of the diagnosis, ie the concordance of MS in northern monozygotic twins was greater than southern monozygotic twins by two times [23]. The risk of MS in the co-twin is increased by two times when the affected twin had early disease, that is before the median age which is 29.3 years [23]. These data suggest additional risk factors, including infection, latitude and amount of sun exposure during childhood [22], and there may be hormonal factors [24].

1.3.2 Latitude and sun exposure

The prevalence of MS is more in the northern areas of the northern hemisphere, while the prevalence is more in the southern areas of the southern hemisphere [15]. These changes in MS prevalence with latitude have been attributed to the amount of sun exposure. In a relatively recent study which investigated twins with MS in North America between 1980 to 1992 [22], the sun exposure during childhood was studied. The study controlled to some degree the genetic factors, as part of the sample contained monozygotic twins, thus investigators were able to assess the effect of sun exposure independently. The study reported that degree of sun exposure in childhood reduces significantly the risk of MS. The risk of developing MS was reduced by 25% for each unit increase in the sun exposure index [22].

The UV light has an effect on the immunoregulatory cells [16] and has also an effect on the levels of vitamin D, because most of our body's vitamin D is synthesized in the skin and then activated in the kidneys [25]. The other proportion of vitamin D is obtained from food, especially seafood. Therefore, vitamin D deficiency can be seen due to less sun exposure, in

people with dark skin colour who absorb less UV light, and due to malabsorbtion [25]. Vitamin D has anti-inflammatory effects and down-regulates inflammatory markers [25]. This has been confirmed by treatment of EAE mice with an active form of vitamin D, which resulted in complete inhibition of the progression of the disease [26]. Surprisingly, Vitamin D levels were found to be higher in the north European countries. This may be partly explained by the fair skin colour in northern Europeans, whose skin absorbs more UV light [25].

1.3.3 Hormonal factors

Hormonal factors may play a role in MS pathology, because females are affected more than males, and the concordance in monozygotic twins is more when the twins are female [23]. Additionally, MS has a reduced relapse rate during pregnancy and an increase in relapses occurs after delivery [24]. A recent study investigated the effect of environmental factors on the sex ratio of MS (ratio of F: M) and was conducted on Canadian immigrants. The study reported increase in sex ratio among immigrants which is also affected by the origin of the immigrants [27]. The study concluded that environmental factors have greater effects on females than males.

1.3.4 Viral infections

Another proposed contributory factor in the aetiology of MS is believed to be viral infection. Animal studies demonstrated a model of virally-induced CNS demyelination [28]. It is proposed that a viral infection acts as a triggering factor in initiating the cascade of inflammatory reactions in MS subjects. Triggering the immune response in MS and other autoimmune diseases is probably due to molecular mimicry with one of the virus components, which is believed to be a peptide [11].

The relation between viral infection and MS has been appearing in literature for a long time [29]. A number of viruses has been suggested as playing a role in MS aetiology, including

poliomyelitis [30], measles [31, 32], herpes simplex virus [33], Epstein-Barr virus (EBV) [34], parainfluenza 1 virus [35], HHV6 and multiple-sclerosis-associated retrovirus [36].

Among the suggested viruses, EBV may play an important role in causing MS. For recent reviews refer to Pohl 2009 [37] and Salvetti et al 2009 [38]. EBV is a DNA virus that affects 90% of the population worldwide. It causes infectious mononucleosis and may result in encephalitis [37]. There is serological evidence that previous EBV infection may increase risk of MS in children and adults [38]. Several epidemiological studies have shown a relation between EBV infection and development of MS [28].

1.3.5 Other environmental factors

MS geographic distribution generally corresponds to the distribution of developed countries and the economic status of the country [16]. For example, the disease is highest in Japan, which is located in a relatively low risk area (Asia). Two studies reported increase in prevalence in the Middle East; a study reported that the disease prevalence is increasing in Kuwait. This increase was mainly among Kuwaiti females [13]. Similar results were obtained from Jordan, where the prevalence of the disease has increased in the last three decades [14]. Increase in MS prevalence rates in these areas has been related to many factors such as pollution, exposure to solvents, dietary factors, smoking, and change in the amount of sun exposure [16].

1.4 Clinical presentation and disease course

Relapsing remitting Multiple sclerosis

MS follows a relapsing remitting course in 80% of cases and is called relapsing remitting MS (RRMS). Patients with RRMS experience relapses in the form of neurological symptoms that develop over several hours to few days and continue for several days to few weeks. A

patient with RRMS is most often a female in her early 30s, who presents with rapidly progressing symptoms. The ratio of female to male is approximately 2:1 [7].

In 15% of patients, the first symptom is optic neuritis [7]. Patients usually complain of unilateral blurring of vision and gradual onset of pain in the affected eye with no redness. Examination of the affected eye may reveal optic disc inflammation. These distinct features of optic neuritis in MS help in supporting the diagnosis, especially when there is evidence of demyelination in visual evoked potentials (VEPs).

Lower limb weakness in the form of spasticity and hyperreflexia affects 40-60% of cases [7], Fatigue can affect up to 40%, which can be secondary to lower limb weakness and/or depression [39]. Vertigo can affect 20% of cases [40]. Other problems may involve the brain stem, cerebellum, bladder control, sexual function and neuropsychological function.

In most cases symptoms improve with time, and patients may completely recover within a few weeks. It is assumed that these attacks, and the consequent temporary functional failure, are mainly due to acute inflammation and oedema. Remission of these attacks by use of immunosuppressive agents that inhibit inflammation and remove oedema supports this assumption.

Progressive multiple sclerosis

Ten years from onset of RRMS, 40% of cases enter a stage of disease progression [41]. This percentage increases to 80% after 20 years [42]. In this stage, patients accumulate neurological deficits and disability without experiencing remissions. This progressive form of the disease is called secondary progressive MS (SPMS) and is associated with significantly less acute lesions and reduced response to immunosuppressant. The reduced response to

treatment in SPMS may indicate a different mechanism underlying progressive neurological deficits [43].

The other less common form of progressive MS is primary progressive MS (PPMS). This usually affects patients in an older age group. However, patients with RRMS develop progression and the disease transforms to SPMS at an older age than that of those who present with PPMS [44]. PPMS is progressive from onset, and the affected patients accumulate neurological dysfunction over time. Patients with PPMS may have intermittent plateaus, but no clear relapses or remissions [45]. In addition, a considerable number of patients with PPMS experience an episode of MS many years before the onset of the disease [46] or after the disease onset [47].

PPMS has similar incidence in males and females and is characterized by unresponsiveness to immunosuppressant treatment from the beginning. It is believed that the cause of disability in PPMS is mainly irreversible degeneration of axons, in particular small diameter axons [17, 43].

1.5 Diagnosis

Diagnosis of MS is based on clinical features, which are characterised by dissociation in time and space. There is no single test for the diagnosis of MS. Therefore, different diagnostic criteria were established. In 1965, Schumacher established the first criteria for MS. The recent advances in MRI techniques and electrophysiological studies allow scientists to recognise other abnormalities in MS. Therefore, Poser criteria incorporated paraclinical tests such as cerebrospinal fluid analysis (CSF) and evoked potentials (EPs) [48-51].

The recently established McDonald's criteria have replaced both Schumacher and Poser's criteria [52] (Table 1.1). The criteria establish diagnosis of MS earlier than the previous two

criteria. The criteria have been revised in 2005 by Polman et al who explained the definition of the attacks, dissemination and other finding [53].

Table 1.1: McDonald's Criteria

Clinical Presentation	Additional Data Needed
2 or more attacks (relapses) 2 or more objective clinical lesions	None; clinical evidence will suffice (additional evidence desirable but must be consistent with MS)
2 or more attacks 1 objective clinical lesion	Dissemination in space, demonstrated by: MRI or a positive CSF and 2 or more MRI lesions consistent with MS or further clinical attack involving different site
1 attack 2 or more objective clinical lesions	Dissemination in time, demonstrated by MRI or second clinical attack
1 attack 1 objective clinical lesion (monosymptomatic presentation)	Dissemination in space demonstrated by: MRI or positive CSF and 2 or more MRI lesions consistent with MS and Dissemination in time demonstrated by: MRI or second clinical attack
Insidious neurological progression suggestive of MS (primary progressive MS)	One year of disease progression (retrospectively or prospectively determined) and Two of the following: a. Positive brain MRI (nine T2 lesions or four or more T2 lesions with positive VEP) b. Positive spinal cord MRI (two focal T2 lesions) c. Positive CSF

The review by Polman recommended that a clinical relapse or a T2 lesion occurring within one month, instead of 3 months as in the origincal McDonald's criteria, can be usefull in confirming dissemination in time. In addition, spinal cord lesion can be considered to confirm dissemination in space instead of infratentorial brain lesion but not periventricular or juxtacortical lesion. Polman revised the criteria again in 2010, published in 2011. The review recommendations allow for a more rapid and more sensitive diagnosis. It has been agreed that a follow up scan to confirm dissemination in time is not needed if the baseline MRI demonstrated both gadolinium-enhancing and nonenhancing lesions. This simplifies the diagnosis of MS without affecting accuracy and also reduces the MRI examination requirements [54].

1.5.1 Radiological abnormalities (MRI)

MRI techniques are highly sensitive in detecting MS lesions and considered as the major paraclinical test in MS [55-57]. MRI is superior to all other measures in detecting subclinical lesions, 'silent lesions' [55, 56]. Acute plaques appear hypertense in T2 weighted images and proton density weighted images as they reflect a large amount of water, while chronic plaques are hypotense in T1 weighted images, 'black hole'. The black holes are more pathologically specific for MS [55].

Nevertheless, these changes identified by MRI are not disease-specific. Several diseases cause WM lesions that are similar to those of MS, such as ischaemic vascular diseases, normal aging process, acute disseminated encephalomyelitis, lacunar infarcts and leukodystrophy. Therefore, the initial important step in radiological diagnosis of MS is to rule out other similar diseases [49, 50, 58].

Lesions of vascular disease usually do not affect the corpus callosum and tend to be more peripheral (unlike MS). Those hyperintensities on T2-weighted images of the ageing process are smaller, tend to be symmetrically distributed, and are not usually visible in T1-weighted images. Acute disseminated encephalomyelitis lesions are all of similar age, whilst MS lesions are of different ages [56].

1.5.2 Neurophysiological abnormalities

The general principle for neurophysiological studies is the use of electrodes to record the amplitude and the duration of neuronal impulses. This will detect delay or failure of transmission of an impulse in a certain tract. In MS disease, the most important finding is change in latency rather than amplitude, since latency prolongation is more consistent with a demyelinating process [59, 60].

To record abnormal neurophysiological response, there should be sufficient damage in the examined tract. In addition, not all the tracts can be explored by these studies. Therefore, the correlation between neurophysiological study results and the degree of impairment is quite poor [60].

The most commonly used methods in MS are the stimulus-related EPs. The currently used stimulus-related EPs in the diagnosis of MS include visual EPs, auditory EPs, brain stem auditory EPs, and somatosensory EPs.

The highest sensitivity is obtained from visual EPs, where a delay in latency is demonstrated in most cases with optic neuritis [51, 59]. The diagnosis of delay in latency of the optic nerve is established by considering the difference between the two eyes and not by measuring the absolute values [59, 60]. A difference of more than 10 milliseconds in latency is abnormal even if both readings are within the normal range [5].

The sensitivity of auditory EPs in evaluating the auditory system, when complemented with brain stem auditory EPs, can reach 83%. For motor EPs, the sensitivity to detect central corticospinal spinal tract (CST) damage is low. This is due to the wide range of normal values [59].

1.5.3 Cerebro-spinal fluid analysis

A CSF sample is frequently obtained during the diagnostic process in MS. The aim of analysis is to look for intrathecal markers that are most peculiar to MS. Oligoclonal bands are the most specific CSF changes for MS, followed by intrathecal IgG synthesis [61, 62]. Both indicate increase intrathecal synthesis of immunoglobulins. However, these changes can be detected in other types of chronic CNS inflammation, such as neurosarcoidosis, subacute sclerosing panencephalitis, and primary CNS lymphoma [61].

1.6 Pathophysiology of multiple sclerosis

The principal lesions of MS are the plaques. The WM plaques tend to be round or ovoid in shape. In the brain, they are commonly peri-venular. MS can result in demyelination in the cerebral cortex and in the deep grey matter (GM) nuclei of the brain and the spinal cord. Initially MS causes swelling of the nervous tissue but, with progression of the disease, older lesions possibly shrink, and CNS atrophy is a prominent feature. It has been suggested that in acute MS the mechanism of injury is mainly inflammatory, while with chronic MS, neurodegeneration is the main mechanism [63]. In summary, stages of the plaque development include inflammatory loss of OLs and myelin, partial recovery, post-inflammatory gliosis, and neurodegeneration [11].

1.6.1 Inflammatory loss of oligodendrocytes and myelin

The inflammatory aspect in the pathology of MS disease was first described long ago. In fact, peri-vascular inflammatory cells were demonstrated when the disease was described for the first time by Charcot [43]. Inflammatory reaction in MS was then suggested to be autoimmune in nature in 1933 by demonstration of demyelination in EAE [64].

A great advance in the understanding of MS immunology comes from EAE [65-67]. It is the most commonly used animal model for MS [65, 66]. By examining EAE scientists were able to identify important disease markers, such as oligoclonal bands [5], which is used for supporting the diagnosis of MS [5, 65].

One of the earlier steps in the MS inflammatory process is thought to be migration of T cells from the systemic circulation to the CNS through the blood brain barrier (BBB). These T lymphocytes (CD4) are activated, possibly by antigen [17].

In the CNS side, there is up-regulation of a number of molecules, such as inter-cellular adhesion molecule 1 (ICAM 1) and vascular adhesion molecule 1 (VCAM 1), and proteases. These molecules assist in adhesion and migration of T cells into the CNS [17]. This up-regulation of adhesion molecules, and other molecules, is possibly caused by signal from the activated T cells themselves, via secretion of interferon Gamma (IFN-γ) and tumour necrosis factor Beta (TNF-β) [17, 63].

The abnormally active T lymphocytes (CD4) affect the permeability of the BBB. Consequently, these cells can penetrate the BBB to the CNS [5, 43, 65]. This may explain the observation that the disease plaques are found mainly around small veins, 'peri-venular'.

T lymphocytes or CD4 cells are divided into T helper cells 1 (Th1), T helper cells 2 (Th2), T regulatory cells and the more recently identified T helper 17 (Th17) [68]. In the CNS, CD4 Th1 cells secrete IFN-γ, interleukin 2 (IL2), TNF-β, and other cytokines [7, 65, 66], while Th17 cells secrete tissue destructive cytokine known as interleukin (IL17) [68]. These cytokines initiate a cascade of inflammatory reactions, which involve fluid accumulation, 'swelling', and direct damage to myelin sheaths, OLs, and axons.

In this acute phase of the lesion, there is evidence of active myelin destruction as seen by the electron microscope (macrophages filled with myelin breakdown products) [5, 65, 67]. There is also evidence of axonal loss in the plaque and the normal-appearing areas [69], which can be primary axonal loss or secondary to loss of myelin and OLs. However, researchers commonly describe axons as relatively preserved in MS compared to loss of OLs and myelin. Magnetic resonance spectroscopic (MRS) changes in N-acetylaspartate, a marker of the neuronal function, have been noted in the plaques of MS, which may indicate that there is also loss of function of surviving axons. Furthermore, EPs reveal a delay in

latency in the affected tract [59, 60]. Consequently, these acute lesions are believed to be responsible for neurological dysfunction (clinical relapses).

1.6.2 Recovery and remyelination

In RRMS the immune response will be inhibited, in most cases, after a few weeks. T regulatory cells and CD4 Th2 cells are possibly responsible for this inhibition. T regulatory cells secrete interleukin 10 (IL10) and transforming growth factor beta (TGF-β), which are major cytokines suppressing the immune reaction. CD4 Th2 cells down regulate the immune response by secreting different cytokines such as interleukin 4 (IL4), interleukin 5 (IL5), interleukin 13 (IL13), and possibly TGF-β [65, 66].

As time passes, signs and symptoms of the disease start to improve and the patient enters a stage of recovery. During this period, examination of some plaques shows reformation of thin myelin sheaths, which does not fully explain the degree of recovery [5, 66]. The number of OLs in lesions correlates with the degree of remyelination. Presence of remyelination and the clearance of the accumulated fluid and neurotoxins may allow the axons to retain part of their conduction capacity [43, 66]. This period of recovery refers to the remission of the relapse which is observed clinically in RRMS. In comparison, PPMS and SPMS do not show complete remission and, therefore, the mechanism of tissue injury and/or the mechanism of tissue repair in these forms may be different or defective. Nevertheless, with progression of the plaque, this repair is limited by the action of astrocytes by producing gliosis (scarring) [11, 43, 66].

1.6.3 Post-inflammatory gliosis

Following demyelination and tissue injury, astrocytes become activated. Astrocytes are activated by signals released in the site of the CNS injury [70]. The reactivated astrocytes migrate to the lesion. These activated astrocytes are responsible for the formation of the glial

scar, by mediating inflammatory reaction and remodelling of the lesion. This is characterised by hypertrophy of astrocytes, increased expression of glial fibrillary acidic protein (GFAP), and cellular proliferation. Many believe that the astroglial scar inhibits axonal growth and regeneration. However, a subset of astrocytes may act as progenitor cells, and thus may have neuroprotective functions [70, 71].

1.6.4 Neurodegeneration and axonal loss

The long-term effects of scarring may include neurodegeneration. There is a reduction in the number of surviving axons in the areas surrounding lesions, which indicates that there is a significant number of severed axons [72]. This may result in degeneration of axons segments in areas far from the primary lesion via the effect of neuronal degeneration, which may continue for months or years after injury [11]. Furthermore, neuronal degeneration may occur due to environmental changes around the axons or the neurons, such as loss of the myelinating OLs.

1.7 Nervous tissue injury

This section discusses the normal CNS response to injury and types of consequent neuronal degeneration. This will provide a better understanding of the possible mechanisms of axonal and neuronal degeneration in MS, especially in the spinal cord. This is because the spinal cord contains both ascending and descending tracts, which have connections with the brain.

1.7.1 Neuronal injury

Apart from specific areas in the brain, such as the hippocampus and the olfactory bulb, neuronal precursors do not divide, and thus no new neurons are spontaneously produced following injury [73]. Neurons in the CNS are frequently exposedvulnerable to different types of traumas including neurotoxin exposure, infection, mechanical and neurodegenerative trauma. Such trauma can be tolerated by neurons with the help of microglia. Microglia have

an intimate relationship with neurons and have both neuroprotective and, at times, neurodegenerative roles. In minor neuronal insult, microglial cells protect injured neurons by supplying them with neurotrophic factors, such as neuronal growth factor and TGF-β [74, 75].

Initially, microglia do not express any phagocytic or cytotoxic activity against injured neurons, but rather respond to injury by proliferation and secretion of more neurotrophic factors, in an attempt to protect the injured neuron. Microglial cells can also cause de-afferentation of the injured neurons, which protects neurons from further damage by the excitatory synapses, such as glutamate [76, 77]. De-afferentation facilitates exchange of neurotrophic factors between neurons and microglia, by increasing the exposed surface area of the neuron. However, prolonged de-afferentation may result in degeneration [75, 76].

Neuronal phagocytosis occurs when the neurological insult is beyond the ability of the neuronal tissue to protect itself. At this stage, microglia express phagocytic activity against neurons. Response of microglial cells depends largely on signals from the injured neurons themselves. Microglia will then phagocytose debris of terminally damaged neurons [78].

1.7.2 Axonal injury

At the axonal level damage can be induced by different types of insult, including ischaemia, low or high temperature, direct pressure or cutting, neurotoxins, and infection. (For review refer to Fitch and Silver 2008 [74]). Some of these insults are noticed in MS such as the effect of temperature on the function of axons. MS patients commonly experience worsening of symptoms after a hot bath such as blurring of vision and fatigue (Uhthoff's sign). There is also probable effect of pressure caused by the oedema on the axonal functions [79].

Generally the peripheral nervous system (PNS) axons have better ability to regenerate than the CNS axons, due to presence of trophic factors that promote regeneration in the PNS. Injured CNS axons grow slowly and do not cross the site of injury forming the so called retraction bulbs. In the PNS, Schwann cells promote regeneration and guide axons to their targets, while the equivalent myelinating cells of the CNS (OLs) appear to inhibit axonal regeneration. Evidence of the critical role of peripheral glial cells in enhancing regeneration was shown by David in 1981, who demonstrated that CNS axons can regenerate when a peripheral nerve is grafted around them [80].

In the PNS, myelin substance is rapidly cleared by macrophages allowing better healing, while myelin in the CNS is slowly cleared, due to late appearance of macrophages in the site of injury [81, 82]. This fact indicates that the myelin substance may inhibit axonal growth, and that there is a critical role of the BBB in preventing recruitment of macrophages to clear the inhibitory myelin from the site of injury [82]. One of the earliest applications of this fact is the use of anti-CNS myelin monoclonal antibody (IN-1), which stimulated axonal growth in experimental animals and improved neurological recovery [81]. Later on, the antigen of this antibody was found to be Nogo-A, which will be discussed later in this thesis [83].

1.8 Neuronal degeneration

Anterograde (Wallerian) degeneration

For a normally functioning neuron, continuity between the neural cell body and the axon should not be disrupted. The axon depends largely on the cell body for survival. Therefore, in the CNS and the PNS, the distal segment of the severed axon degenerates. This phenomenon was observed by Augustus Waller in 1850 [84], and thus named Wallerian degeneration. The process of Wallerian degeneration is considered a normal response of the body to damaged or unnecessary axons, and it is vital in normal development of the CNS and for regeneration of the severed axons in the PNS [85].

Axons do not synthesize their own nutrients, but rather depend on special transport mechanism of proteins from the cell body along the nerve fibres [85]. However, starvation of the axons, by not being connected to the cell body, cannot be the direct cause of Wallerian degeneration since axonal degradation happens within a few days of axonal transection. Previous studies showed that Ca+ is the main factor in inducing Wallerian degeneration. Wallerian degeneration did not happen in the nervous tissue grown in low Ca+ media. Moreover, calcium channel blockers, such as cobalt and manganese, prevented Wallerian degradation in tissue culture [85]. Within days, presynaptic terminals of the severed axons retract from the postsynaptic neuron and from the presynaptic neuron. Following retraction of the presynaptic terminal, the distal segment degenerates by Wallerian degeneration [82, 86].

Stages of Wallerian degeneration are divided into four periods; the lag period, period of entry of Ca^{+}, period of activation of Ca^{+}-dependent proteases, and period of degradation of the axon. The lag period is variable among different species. It lasts for 24-48 hours in rats and up to 7 days in humans [85].

The first observation in Wallerian degeneration is granular disintegration, which is thought to be a Ca+-induced phenomenon via activating tissue proteases. This early stage happens in both the CNS and the PNS with the same timing, but later stages are much slower in the CNS [82]. Once the axon is damaged, Ca+ enters the axon and activates tissue proteases, which are enzymes that accelerate degeneration [85].

In the CNS all features of Wallerian degeneration, including axonal degradation, macrophage activation and myelin phagocytosis, continue for up to 90 days post injury. In the PNS, degeneration maximally takes 30 days to complete (animal study) [82]. A study by Rachel George in 1994 investigated Wallerian degeneration in rats, by transecting primary

sensory neurons at L4, L5, and L6 levels. The level of cut was just proximal to the dorsal root ganglia, which allows for studying Wallerian degeneration of the same axons in both the CNS and the PNS. The study revealed that the varaition between the CNS and the PNS in degeneration is due to the different environment. It demonstrated that the part of the axon that runs in the CNS shows the same characteristics of Wallerian degeneration as the purely CNS axons, and the part of the same axon that runs in the PNS degenerates similarly to peripheral axons. The author suggested that this may be due to delayed recruitment of macrophages as a result of the BBB [82]. This has been also supported by another study, which demonstrated enhancement of regeneration in the optic nerve by covering it with macrophages [87]. It is important to note that in the CNS and the PNS, the ability to regenerate axons declines with age [88].

Retrograde degeneration

The proximal part of the axon, which remains connected to the neuronal cell body, usually does not degenerate initially and neurons may survive for years, especially when the site of injury is far from the cell body [89]. However, some changes in the cell body have been noticed following cutting the axon, therefore, retrograde degeneration affects the neurons and the axons.

In fact, retrograde degeneration involves redistribution of intracellular organelles, rather than actual degeneration. Retrograde degeneration includes swelling of the neuronal cell body, redistribution of Nissl bodies on the periphery, increase in ribosomes and protein synthesis, and the nucleus acquires an eccentric position. These changes reflect increase in metabolic activity and indicate that the cell is ready to re-innervate during this period [90]. These changes are sometimes called chromatolytic changes and were reported after axotomy by 24-48 hours [91]. They are not specific for neurons with severed axons, but can also occur

after other forms of neuronal injury: for example, they have been reported in alcoholic encephalitis [92] and in Werdnig-Hoffmann disease [93].

If time passes and the axon fails to regenerate and re-innervate, the cell body will probably die. In MS, different studies have suggested the potential role of retrograde degeneration in neuronal loss in areas far from the primary lesion [94, 95]. In both directions (anterograde and retrograde), degeneration may continue to the nearby synapses and neurons in a process called transynaptic degeneration [96].

Transynaptic degeneration

In long pathways, where there is more than one order neuron, degeneration may involve the entire pathway in a process called transynaptic degeneration. Degeneration of the pathway continues in both directions (anterograde vs retrograde). For example, transynaptic anterograde degeneration may occur when the retina is damaged, degeneration affects the pathway up to the visual cortex including the lateral geniculate nucleus. This has been suggested in a study on the lateral geniculate nucleus in patients with MS, when there was reduction in the number of neurons in the nucleus. While transynaptic retrograde degeneration occurs when the visual cortex is damaged for example, degeneration may continue through the lateral geniculate nucleus up to the retinal neurons [95, 97].

1.9 The spinal cord in progressive multiple sclerosis

In this project we have examined the spinal cord pathology in progressive MS. The progressive forms of MS are frequently associated with irreversible disability. The two major forms, PPMS and SPMS, have different clinical, radiological, and pathological features. PPMS commonly presents with spinal cord lesions and has been suggested to be a different disease entity [98-100]. PPMS is associated with poorer prognosis compared to SPMS; on average it takes 6 years for PPMS patients to develop severe disability, compared to 11

years in patients with RRMS [101]. Howeve, when RRMS patients enter the phase of progression, the rate of clinical deterioration was found to be similar in PPMS and SPMS [41]. Therefore, the subtype of the disease at presentation (PPMS vs RRMS) is considered the most important factor affecting disease prognosis. Radiologically, there are more brain lesions affecting RRMS and SPMS compared to PPMS [102], and pathologically, there is a greater amount of inflammation [103]. However, most of the previous studies have been conducted at the level of the brain and few studies have been carried out on the spinal cord GM [104-108]. Recognition of the importance of spinal cord pathology in studying MS disease has increased recently. Based on the recent McDonald criteria, incorporating spinal cord MRI with brain MRI in the diagnosis of MS increases the sensitivity of diagnosis up to 100% [53, 109, 110]. Atrophy of the spinal cord in progressive MS has been confirmed previously [105, 111, 112]. This atrophy correlates significantly with disability [113-116]. MRI measurement of spinal cord atrophy was also used as an indicator to follow the disease response to treatment [117-119].

Furthermore, lesions of the spinal cord commonly involve the WM and the GM which gives the examiner the ability to compare pathology in the GM and the WM within the same lesion, same subject and same level [105, 120, 121]. Similarly, ascending tracts and descending tracts can be found in the same lesion and thus their pathology can be compared [122].

More interest has arisen during the last decade in studying the mechanism and extent of spinal cord pathology [2, 4]. The new interest is evidenced by the number of publications that can be found in Pub Med under these keywords; multiple sclerosis; brain, gray matter or grey matter, and spinal cord (Pub Med was accessed through the search feature of endnote programme on 12/11/2010). The number of articles that has multiple sclerosis in the title was 26270 papers. If the keyword "brain" is added, the number is 889, while the number reduces

significantly when the keyword "spinal cord" is added instead of the keyword "brain" (230 papers).

The number of studies that have the keywords "multiple sclerosis" and "brain" and "gray matter" is 16 studies. On the other hand, only 3 studies were found, when you replace the keyword "brain" with "spinal cord", which were all carried out in our lab [104, 106-108]. Figure 1.1 demonstrates the number of published studies over the last 15 years that has the word multiple sclerosis and gray matter or grey matter in their title. This certainly does not include all the studies that investigated pathology of the GM in MS disease, but it gives a rough comparison between the number of studies in the last two decades.

The data above indicates that there is a significant lack of research in the histopathology of the spinal cord in MS. In addition, there are few studies that investigated the differences in the pathology between disease subtypes at the level of the spinal cord.

1.10 Thesis outline

Using quantitative measures, this thesis examines the histopathology of MS at the level of the spinal cord. Quantification includes the five major CNS changes involved in MS, which are atrophy of tissue, degree of demyelination, OLs loss, axonal loss and neuronal loss. The project investigates the variation in these measures between MS and controls and between PPMS and SPMS. The analysis involves multiple spinal cord segments and is not restricted to the upper cervical levels. This can address the effects of topographic variation on atrophy, demyelination degree, OLs loss, axonal loss, and neuronal loss.

This research has been carried out by studying human autopsy material of both controls and MS patients. For the next chapter, the degree of atrophy and demyelination of the spinal cord was investigated in PPMS and SPMS using the MBP antibody.

In Chapter 3, optimisation of a reliable quantification process of OLs was established. For Chapter 4, the unbiased quantification process of OLs was applied to assess numbers of OLs in the ventral horn (VH) and the dorsal horn (DH) of the spinal cord, while for Chapter 5 we quantified OLs in the spinal cord WM.

Quantification of axons was carried out for Chapter 6 using immunohistochemistry with NE14 marker. The quantification process of axons was similar to the protocol applied on quantification of OLs in the WM, ie quantification of axons and OLs was carried out in topographically the same fields. Chapter 7 investigates numbers of neurons in the VH. Chapter 8 summarises the thesis and includes suggestions for future work.

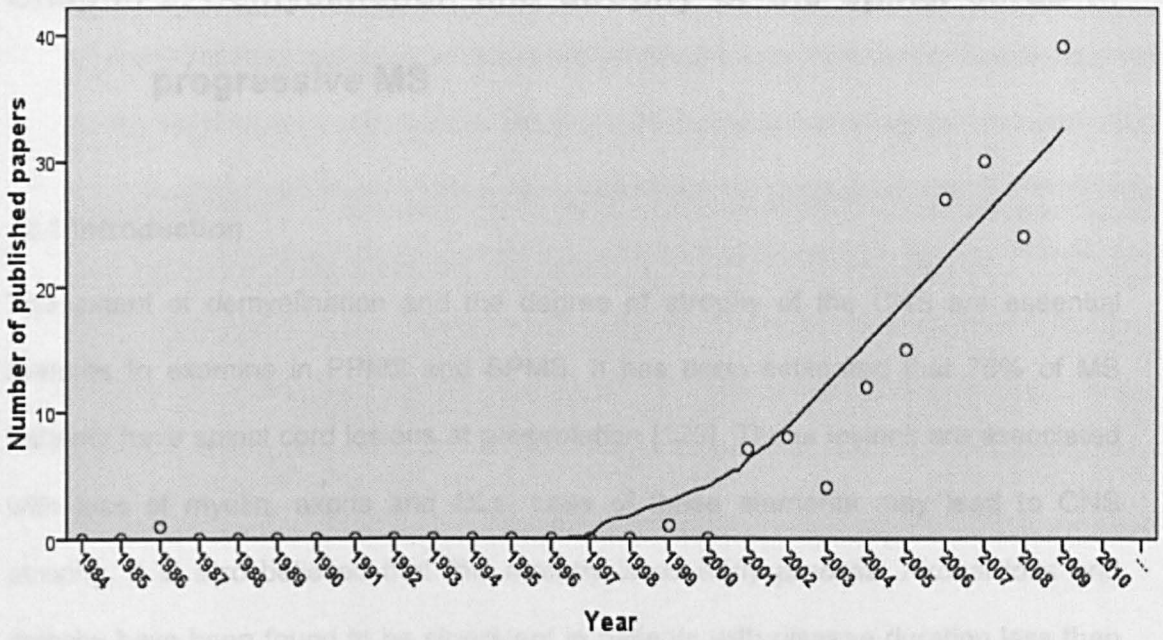


Figure 1.1: The number of studies that have multiple sclerosis and grey (or gray) matter as the key words. Pub Med was accessed on 12/10/2010.

Chapter 2: Demyelination and atrophy of the spinal cords of progressive MS

2.1 Introduction

The extent of demyelination and the degree of atrophy of the CNS are essential features to examine in PPMS and SPMS. It has been estimated that 75% of MS patients have spinal cord lesions at presentation [123]. These lesions are associated with loss of myelin, axons and OLs. Loss of these elements may lead to CNS atrophy. It is also believed that this atrophy is an early process. Axonal loss and atrophy have been found to be significant in patients with disease duration less than 5 years [111].

The exact mechanism of spinal cord atrophy in MS is still unknown. The relation between amount of demyelination and atrophy is not clear. Different mechanisms and theories have been suggested to explain nature of atrophy. An earlier histopathology study showed that local effects of lesions on atrophy are probably limited [112]. Conversely, in an MRI study, degree of atrophy was found to be significantly affected by degree of spinal cord lesions [115]. A histopathology study has also reported significant positive correlation between atrophy and demyelination [124]. Histopathological studies are well known as being more accurate than MRI studies in assessing the cord cross sectional area (CSA) and degree of demyelination [125].

2.2 Volume of the GM in multiple sclerosis

Few studies have investigated the degree of atrophy and degree of demyelination in the GM of the spinal cord. While GM atrophy of the brain has been confirmed previously, GM atrophy of the spinal cord has not yet been confirmed. There is also

some evidence that spinal cord pathology is independent of brain pathology because neither atrophy nor occurrence of lesions correlates [126].

Two previous histopathology studies examined potential atrophy of the spinal cord GM and they showed opposite results. The first study was carried out in 2000, and reported that spinal cord atrophy was due to WM and GM atrophy to the same degree, and that the GM:WM ratio of MS subjects was similar to controls even with the presence of significant atrophy [127]. Later, in 2005, Gilmore et al reported in a histopathology study that atrophy is purely due to loss of the WM volume and that GM volume is well preserved in spinal cords of subjects with MS [105]. The later study in 2005 was well controlled and utilised a larger sample size (55 MS and 33 controls compared to 5 MS and 6 controls).

Gilmore and his colleagues attributed the preservation of the spinal cord GM in MS to the discrepancies in connectivity of the examined GM regions. Regions of the GM that have extensive connections with various structures of the CNS, such as the thalamus, are more vulnerable to atrophy than other regions. This fact has been also stated in other studies [105, 128, 129].

The effect of distant lesions via transynaptic retrograde or anterograde degeneration is one of the presumed mechanisms in atrophy of the GM areas with extensive connections [130, 131]. The reported WM atrophy and preservation of the GM in the spinal cord is further supported by the finding that spinal cord atrophy is minimal in the lower cord where the WM content is small. Therefore, understanding the discrepancy in nature of atrophy between the brain and the spinal cord may be very useful in explaining mechanisms of atrophy.

While the clinical significance of spinal cord GM pathology has not been confirmed yet, a number of studies has demonstrated the clinical significance of GM atrophy in the brain [132-134]. For example, GM atrophy of the brain seems to be more related to progression of the disease than WM atrophy [134] and GM disease dominates the pathological process of MS in the progressive phase of the disease [132]. Correlation of brain GM atrophy with disease disability has also been reported previously [132, 134] and even reported to reflect disability better than WM atrophy or WM lesions [134]. In a study executed on PPMS, degree of cerebral GM pathology was found to be a significant predictor of disability over 5 years of follow up [135]. In SPMS, a cross-sectional study showed that decline in the brain volume has a significant effect on patient disability [136].

2.3 Variation between primary progressive and secondary progressive multiple sclerosis

Although the two major forms of progressive MS (PPMS and SPMS) share a similar degree of neuropsychological disability [137], they are different in other aspects, such as disease course, extent of demyelination and inflammation [103, 138], total number of lesions, rate of occurrence of new lesions [138, 139], and degree of atrophy [102].

2.3.1 Variation in degree of demyelination and inflammation

A study by Foong in 2000 analysed 12 patients with SPMS and 13 patients with PPMS. The study demonstrated more lesions in the brains of SPMS compared to PPMS, but the neuropsychological deficits did not show significant difference [137]. Comi et al (1995) studied 14 patients with PPMS and 17 patients with SPMS for variation in brain MRI and relation to cognitive functions. Lesions were significantly more extensive in the brains of SPMS compared to PPMS, especially in the frontal

and occipital lobes. In addition, SPMS patients showed more neuropsychological abnormalities than PPMS [140].

The significantly higher frequency of enhancement with gadolinium in new lesions of SPMS compared to PPMS is a good evidence that degree of inflammation may vary among the two forms [139]. Revesz et al (1994) conducted a human pathological study on 9 cases of MS (4 PPMS and 5 SPMS). The study reported a significant difference in the degree of demyelination and inflammation in PPMS and SPMS. Degree of demyelination and inflammation was much higher in the brains of SPMS than PPMS, but there was no difference at the spinal cord level [103].

Longitudinal studies have also reported differences between the two disease subtypes. In an MRI study that followed up progressive MS patients over one year, imaging of the brain and the cord showed that 85% of new lesions occurred in the brains of SPMS patients, with much fewer new lesions affecting the PPMS patients. New spinal cord lesions formed only 5% of the total number of new lesions, with no difference between PPMS and SPMS [138]. It was also found that PPMS is associated with diffuse abnormality, but SPMS causes more focal lesions of the cord [102].

Another MRI study by Thompson et al (1991) showed that rate of occurrence of new lesions is 18.2 lesions per patient per year in SPMS, compared to 3.3 lesions per patient per year in PPMS. However, the clinical deterioration over the follow up period did not show significant differences between PPMS and SPMS [139].

There is little information about the differences between PPMS and SPMS at the level of the spinal cord, and the pathology study by Revesz investigated a small

sample size (4 PPMS and 5 SPMS). MS lesions of the spinal cord may have better correlation with disability than brain lesions, because higher numbers of new lesions in the brains of SPMS subjects were not associated with significant difference in the clinical deterioration between PPMS and SPMS [139]. This may indicate that the clinical deterioration over time correlates with new spinal cord lesions rather than brain lesions. Nonetheless, the better correlation of the spinal cord pathology with signs and symptoms of MS has been suggested previously [110, 141].

2.3.2 Variation in atrophy

Degree of spinal cord atrophy is apparently not similar in PPMS and RRMS. Nijeholt et al (1998) used various MRI parameters of the brain and the spinal cord to differentiate between MS subtypes. They found that SPMS had more spinal cord atrophy than RRMS and PPMS [102].

A recent longitudinal MRI study conducted by Fisher et al (2008) followed 17 healthy control subjects, 36 RRMS, and 27 SPMS patients for 4 years. Both GM and WM atrophy were measured on a number of occasions. Fisher and colleagues reported that brain WM atrophy affects the three groups to the same degree, but GM atrophy rate varies among the groups and was most prominent in SPMS. The study also revealed significant correlation between tissue damage and GM atrophy in RRMS. In SPMS, there were no predictors of GM atrophy, which indicates that atrophy in the progressive form of the disease continues regardless of age and gender and, most importantly, regardless of lesion load. However, rate of brain GM atrophy correlated significantly with development of disability over the period of follow up [132]. Although the study did not include PPMS subjects, it gives an idea about the pattern of atrophy in RRMS and SPMS. Therefore, it is important to investigate if there is any difference in degrees of atrophy and demyelination of the spinal cord between PPMS and

SPMS. In this study, post-mortem analysis of a large sample of spinal cords from both PPMS and SPMS was carried out.

2.4 Aims and hypothesis

Spinal cord atrophy correlates with disability in MS patients and can be used to follow up treatment strategy [116]. In spite of MRI development in estimating spinal-cord atrophy, the small size of the lower cord restricts accurate estimation of atrophy and demyelination in the lower cord levels due to MRI resolution limitation. In order to precisely assess the dynamics of spinal cord lesions and atrophy, accurate measurement of these parameters in multiple cord levels is essential.

In addition, pathological studies have demonstrated greater accuracy in assessing degree of spinal cord demyelination. Gilmore et al 2009 reported less accuracy in identifying demyelination by in vitro MRI compared to histopathology. This was more obvious in the GM [108]. Such findings may direct future MRI techniques toward improving GM imaging. Therefore, post-mortem pathological studies are helpful in validating MRI results and help in improving accuracy of future MRI techniques.

Accurate assessment of atrophy in vivo may enable clinicians to predict disability and response to treatment accurately. MRI studies showed differences in the nature and degree of atrophy between PPMS and RRMS, mainly at the level of the brain. There is little information about differences in the degree of atrophy and demyelination between PPMS and SPMS at the spinal-cord level.

In this study, MS and healthy human autopsy material will be analysed by measuring the CSA of the spinal cord section. Results will be compared between MS and controls and between PPMS and SPMS. Same analysis will be applied on the GM

and the WM sectional areas separately, to assess the contribution of GM and WM in the atrophy. The effects of independent factors such as age, gender, disease duration, disease subtype and cord level on the cord CSA will be assessed. The demyelinated regions will be measured. Absolute lesion size (the cross-sectional areas of the lesion) and proportion of lesion area to the CSA of the WM and the GM will be calculated.

2.5 Material and methods

Human spinal cords autopsy material was available from three sources: the MS Society Tissue Bank (Imperial College, London) (PPMS = 12, SPMS = 15, Controls = 5), Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam) (PPMS = 7, SPMS = 23, Controls = 5), and Oxford Radcliffe NHS Trust (Controls = 6).

Considering the whole sample of tissue: the age range of PPMS subjects (n = 19; 6 males and 13 females) is between 45 and 92 years (mean ± SD = 67.8 ± 13.4 years) and the disease duration between 5 and 54 years (mean = 25.6 ± 14.5 years); for SPMS subjects (n = 38; 11 males and 27 females) the age is between 34 and 85 years (mean = 58.4 ± 13.6 years) and the disease duration between 4 and 54 years (mean = 26.0 ± 14.3 years); for Control (n = 16; 6 males and 10 females), the age range is between 33 and 93 years (mean = 67.3 ± 3.2 years).

The spinal cords were dissected after a post-mortem delay of 8 to 33 hours for Controls (mean = 18.1 ± 8.4 hours), 5 to 21 hours for PPMS (mean = 10.4 ± 5.3 hours) and 4 to 28 hours for SPMS (mean = 10.4 ± 6.0 hours). For several cases, the post-mortem delay was not available (Tables 2.1, 2.2, and 2.3). Consent to obtain tissue for research purposes and to access medical records was taken from the

donor or the next of kin. Detailed clinical information is available for most cases. Diagnosis of MS has been pathologically confirmed for all cases. The clinical disability preceding death for the two groups (PPMS and SPMS) was found to be similar in a previous study, where EDSS was found to be greater than 8 for majority of cases [142]. The study was approved by the Local Research Ethics Committee.

Table 2.1: The controls sample

Source of tissue	Subject ID number	Gender	Age (Years)	Post-mortem delay (Hours)	Available spinal cord segment
Oxford controls	272	female	54		Cervical, lumbar
	414	female	66		Cervical, lumbar
	458	female	68		Cervical, Lumbar
	3269	male	77		Cervical, lumbar
	3313	male	50		Cervical, lumbar
	4254	male	33		Cervical (not included in analysis), lumbar
Tissue bank controls	11	male	77	26	Lumbar
	14	female	64	18	Cervical, lumbar
	22	female	69	33	cervical
	32	male	88	22	Lumbar
	080	female	93	9	Thoracic, Lumbar
Dutch tissue controls	45	female	41	14	cervical
	142	male	73	25	cervical
	250	female	68		cervical
	270	female	72		cervical
	400	female	83	8	Cervical

2.5.1 Initial preparation of tissue

MS society tissue bank sample

MS Society tissue bank sample includes frozen blocks from various levels of the cord. From 15 cases of MS subjects and 2 cases of controls we have two blocks, while from the other cases we have only one block from each subject. Therefore, 49 blocks (7 controls and 42 MS) from the MS Society tissue bank were analysed. After dissection, all spinal cord blocks were fixed immediately in 4% paraformaldehyde for 2 weeks, then cryoprotected with 30% sucrose for one week. This was followed by immersion in isopentane on a bed of dry ice. From that time, all blocks have been stored at -75 degrees. Blocks were then defrosted at our laboratory and formalin-fixed paraffin-embedded blocks were produced. The formalin-fixed paraffin-embedded sections were cut at a thickness of 5 μm, mounted on Superfast slides, and dried overnight at 37°C.

Netherlands brain bank

A collection of 35 sections of tissue from Netherlands brain bank had been fixed in 10% formalin for 30 days. Tissue material was taken from the cervical cord. Similarly, 5 μm thick sections were cut from the formalin-fixed paraffin-embedded blocks, mounted on Superfast slides, and dried overnight at 37°C.

Oxford Radcliffe Hospital Brain Bank

Six controls were selected randomly from archival material that had been obtained originally from the neuropathology department of Oxford Radcliffe NHS trust. From each control subject, two blocks were selected for analysis; one is cervical and the other is lumbar. Similarly, the formalin-fixed paraffin-embedded sections were cut at 5 μm, mounted on Superfast slides, and dried overnight at 37°C. However, one cervical section was not used because of partial tissue loss during sectioning. Therefore, 11 sections from the 6 controls were included in the analysis.

2.5.2 Immunohistochemistry

The sections were deparaffinised using xylene and rehydrated in reducing concentrations of ethanol (100%, 96%, 70% and water). Endogenous peroxidase was blocked using 0.3% H2O2 in methanol. For antigen retrieval sections were heated in Tris/EDTA (pH 9). The immunohistochemistry staining used primary antibody against myelin (MBP, dilution 1:200, DAKO). The sABC method for immunohistochemistry was performed as previously described in Geurts et al 2005 [143].

2.5.3 Image analysis

The MBP-stained slides were numbered so that the primary observer is blinded to the disease type. All slides were scanned by the Nanozomer Digital Pathology (NDP,

Hamamatsu, Japan). The NDP machine converts the slides into digital slides by scanning the slides at specified magnification. All our slides were scanned at magnification of 40X. These digital slides can be viewed by the computer by special software called NDP.view software. The software enables the examiner to view the scanned slides and to magnify them up to 40 times with the same resolution as a real microscope. It also enables the investigator to take accurate calculations of distances, surface areas and boundaries. The software can be used for drawing around various structures, such as the GM and the WM, and export any field of interest as a digital image in the JPEG format [144].

2.5.4 Demarcation of surface areas

The WM and GM boundaries were demarcated in all electronic sections. The GM boundary that we applied in this study ends at the cap of the DH, which is called the posterior marginal nucleus or zona spongiosa. To increase the accuracy of drawing, boundaries of the GM, WM area and demyelination areas were drawn using a digital pen on a graphic tablet (graphic tablet MD 85637 from Medion). The NDP.view software automatically calculates the surface area within the drawn border. Surface areas were measured in mm^2.

The frequency of demyelination of important structures of the spinal cord was estimated, such as the posterior column pathway (PCP), the area of the corticospinal tract (CST), the DH, and the VH. Initially, the PCP was identified in all sections and the area occupied by the lateral CST was identified based on the description mentioned previously [122]. For more objective description, it is the area of the lateral funiculus that is dorsal to a horizontal line passing through the posterior border of GM commissure. Various DH structures were identified based on Rexed description [145, 146]. The aim is to look for involvement of the nucleus proprius. The DH was

considered affected when the nucleus proprius was demyelinated because the substantia gelatinosa is normally unmyelinated area. The nucleus proprius is a heavily myelinated area that forms the bulk of the DH. In all sections, the area of the substantia gelatinosa was initially delineated. The post marginal zone was also demarcated, which is the thin myelinated area dorsal to the substantia gelatinosa and called the cap. Ventral to the substantia gelatinosa, the nucleus proprius was localized.

Table 2.2: The multiple sclerosis sample (MS society tissue bank)

Subject ID	Gender	Type of the disease	Age (years)	Duration of the disease (years)	Post-mortem delay (hours)	Available level
8	male	PPMS	77	28	5	Cervical, lumbar
40	female	PPMS	58	21	6	Cervical, lumbar
44	female	PPMS	45	20	17	Thoracic lumbar
57	female	PPMS	77	31	9	cervical
70	female	PPMS	77	22	21	Cervical, lumbar
81	male	SPMS	72	47	23	lumbar
83	male	PPMS	54	16	12	Cervical, lumbar
102	male	PPMS	73	52	20	lumbar
105	male	SPMS	73	46	8	Thoracic, lumbar
111	male	PPMS	92	54	9	Cervical, lumbar
114	female	SPMS	52	15	12	Cervical, lumbar
127	male	SPMS	51	10	21	thoracic
128	female	SPMS	78	50	22	cervical
136	male	SPMS	40	9	10	Cervical, lumbar
143	female	SPMS	62	19		Cervical, lumbar
147	female	SPMS	60	21	27	Cervical
157	female	SPMS	39	22	12	cervical
162	female	SPMS	58	22	14	cervical
187	female	SPMS	57	17	13	Cervical, lumbar
191	female	SPMS	48	32	28	thoracic
216	female	PPMS	53	5	9	Thoracic, lumbar
218	female	SPMS	56	26	7	thoracic
231	female	PPMS	59	27		Thoracic, lumbar
263	female	PPMS	73	37		Cervical, lumbar
266	female	PPMS	75	8		Cervical, lumbar
298	male	SPMS	72	43	11	cervical
300	female	SPMS	56	34	13	thoracic

Lesions sizes were calculated and classified as pure WM lesions, pure GM lesions and mixed lesions. Then demyelinated areas of the WM were calculated separately from the demyelinated areas of the GM.

2.5.5 Classification of the sections according to the cord level

The main cord level was already known for all sections (cervical, thoracic, or lumbar). The classification process included identifying the cord segment and whether the section was taken from the upper or the lower segment of the main cord level. This has been identified based on morphology of the spinal cross section, which mainly depends on GM morphology [147]. Sections were categorised into five groups; upper cervical (C2-C4), lower cervical (C5-C8), thoracic (T1–T12), upper lumbar (L1-L3) and lower lumbar (L4-L5).

2.5.6 Statistical analysis

All statistics were calculated using SPSS 14. The means of the two disease subtypes were compared using student t-test. Interobserver reproducibility was tested by calculating the coefficient of variation or Pearson correlation (r). Chi-squared test (X2 test) was used to find out the relation between the frequency of demyelination of the spinal cord structures and progressive MS subtypes. For correlation of atrophy and demyelination with disease duration, partial correlation was used controlling for age, gender, disease duration, segment level and disease subtype. Linear regression was used to study the effects of gender, disease duration, and disease type, and level of the cord on the cord CSA and degree of demyelination.

2.5.7 Reliability and reproducibility

Interobserver reproducibility for identification of the cord level depending on morphology of cross section was tested ($r = 0.9$, $p < 0.0001$).The coefficient of variation was used for testing interobserver reproducibility of surface areas measurements. The results of coefficients of variation were 1.7% for the whole CSA, 2% for the WM area, and 6% for the GM area. The two measurements of the demyelinated areas of the WM and the GM had interobserver coefficients of variation

of 4.4% and 9.3% respectively. To study the contribution of different shrinkage factors between London MS tissue and Netherland MS tissue, the CSA of the exact cord level was compared between the two sources ($r = 0.877$, $p = 0.010$). Results are shown in Table 2.4.

Table 2.3: Multiple sclerosis sample (Netherlands brain bank)

Subject ID	Gender	Type of the disease	Age (years)	Duration of the disease (years)	Post-mortem delay (hours)	Available level
21	female	SPMS	70	27	6	cervical
24	female	SPMS	62	29	7	cervical
59	female	SPMS	34	10	7	cervical
73	male	SPMS	77	41	7	cervical
115	female	PPMS	57	19	6	cervical
116	female	SPMS	36	11	6	cervical
121	female	SPMS	45	14	11	cervical
125	female	SPMS	58	20	8	cervical
132	female	PPMS	79	44	8	cervical
155	female	SPMS	85	54	4	cervical
161	female	SPMS	41	7	4	cervical
177	female	SPMS	71	31	10	cervical
189	female	PPMS	82	15	4	cervical
192	female	SPMS	63	32	7	cervical
202	male	SPMS	50	7	6	cervical
228	male	SPMS	70	21	6	cervical
232	female	SPMS	40	4	7	cervical
234	female	SPMS	81	49	4	cervical
248	female	PPMS	57	7	8	cervical
264	male	SPMS	51	29	8	cervical
276	male	SPMS	56	13	5	cervical
282	female	SPMS	76	53	14	cervical
307	male	SPMS	72	42	5	cervical
309	female	SPMS	60	22	9	cervical
323	female	PPMS	70	36	9	cervical
338	male	PPMS	48	19		cervical
352	female	SPMS	53	19	7	cervical
385	female	SPMS	40	18	7	cervical
404	female	SPMS	54	11	7	cervical
3070	male	PPMS	83	37	7	cervical

Table 2.4: Surface area of the cervical segments of MS tissues obtained from the MS society tissue bank and from Netherlands brain bank. Tissue from Netherlands contains only cervical cord tissue.

Level	CSA of the cord (Tissue bank sample)	CSA of the cord (Netherlands tissue sample)
	Mean in mm^2	Mean in mm^2
C2	31.10	28.25
C3	34.53	39.30
C4	30.35	28.20
C5	42.20	41.55
C6	42.00	49.90
C7	50.95	56.74
C8	63.10	54.46

2.6 Results

2.6.1 General observations

We examined 95 MBP-stained sections taken from 73 subjects (29 sections from PPMS, 43 sections from SPMS, and 23 sections from healthy subjects). It was possible to clearly identify the GM from the WM at low magnification for most of the sections. However, when there was extensive demyelination, high magnification was required for delineation of the GM from the WM. Spinal cord areas such as the VH, DH, PCP, and the area of the CST were identified in all sections.

The MBP stain showed the unmyelinated areas clearly. The normally unmyelinated areas in the spinal cords of healthy controls include the part of the intermediate GM around the central canal, the substantia gelatinosa and the outermost area of the lateral horns. These areas were considered when MS slides were examined for abnormality.

In MS, both spinal cord GM and WM were affected by demyelination. Lesions commonly involved the GM and the WM without respecting the boundary. However, lesions seem to extend from the WM to the GM but not the opposite direction, ie lesions frequently acquires the shape of the VH (Figure 2.1).

In the PCP 14 separate lesions were detected. These were midline, bilateral and frequently symmetrical lesions (Figure 2.2). These lesions were seen in both disease subtypes and there was no association between size of lesion and disease duration.

2.6.2 General statistics and distribution of demyelination

Number of sections that showed abnormality was 48, which constitutes 67% of MS sections. About 70% of the cervical sections showed abnormality, compared to 62%

and 58% in the thoracic and lumbar sections respectively. Out of the 43 sections with SPMS, 35 sections (81%) showed abnormal appearance, while 13 sections (45%) from the 29 PPMS blocks showed abnormality.

The CST and the PCP were commonly seen to be affected by complete demyelination. The PCP was affected in 53% of all sections and in 72% of the abnormal-appearing sections, while the CST was affected in 57% of all sections and in 69% of the abnormal-appearing sections.

About 63% of all SPMS sections showed PCP involvement, while in PPMS 37% of sections showed PCP lesions (Pearson chi square = 5.067, $p = 0.024$). The SPMS also showed more frequency of CST involvement than PPMS: 74% of SPMS sections have CST lesions compared to 30% of PPMS sections that have CST lesions (Pearson chi square = 14.253, $p < 0.001$).

With respect to the DH involvement, 52% of SPMS showed demyelination of DH compared to 27% involvement in PPMS (Pearson chi square = 4.846, $p = 0.028$). The VH was affected in 38 sections, which forms 50% of all sections. The VH was also involved more in SPMS, in which 63 % of SPMS sections showed VH lesions, compared to 30% of PPMS sections (Pearson chi square = 7.930, $p = 0.005$). Ten sections showed extensive demyelination that occupied more than 50% of the cord CSA and all these sections were cervical (9 of them are SPMS and only one PPMS).

2.6.3 The spinal cord cross sectional area

The cross sectional areas of MS spinal cords was less than controls

The mean CSA of spinal cords in MS subjects was lower than normal controls in all levels except the thoracic level. In the cervical region, there was significant 31%

reduction in the CSA compared to controls. This decline was more prominent in the upper cervical (46% atrophy, p = 0.001) compared to lower cervical (26% atrophy, p = 0.009). In the thoracic cord, our data showed that the CSA in MS was higher than controls. Since we have only one thoracic block as a control, the comparison between MS and controls cannot be reliable at this cord level.

The lumbar regions of MS showed less atrophy than the cervical levels. Compared to controls, the sectional areas of the upper lumbar and lower lumbar regions were reduced by 20% (p = 0.086) and 29% (p = 0.099) respectively (Table 2.5).

Atrophy of the spinal cords of MS affects the GM and the WM

The amount of WM was reduced by 45% in the upper cervical segment (p < 0.001) and 28% in the lower cervical segment (p = 0.008). Similar results were obtained from the GM measurements, where the cervical GM atrophy was significant in the upper cervical (48% atrophy, p = 0.004) and the lower cervical GM (22% atrophy, p = 0.045).

Results from the lower levels of the cord showed markedly less decline in the CSA of the WM and the GM, which was statistically insignificant except in the GM of the upper lumbar segment (p = 0.049) (Table 2.6).

Table 2.5: The CSA in the 5 examined segments of the spinal cord in MS and controls

Segment Category	Controls and MS					PPMS and SPMS			
	CSA (mm^2)			SD (mm^2)	Sig	CSA (mm^2)		SD (mm^2)	Sig
Upper Cervical	Control	n= 4	58.82	6.03	.001	PPMS	35.76	5.77	.022
	MS	n = 15	31.89	6.31		SPMS	28.50	4.80	
Lower Cervical	Control	n = 8	69.69	14,34	.009	PPMS	55.36	14.23	.388
	MS	n = 32	51.62	13.90		SPMS	50.38	13.86	
Thoracic	Control	n = 1	20.60	....	.154	PPMS	31.43	5.42	.275
	MS	n= 8	28.40	4.86		SPMS	27.30	4.32	
Upper Lumbar	Control	n= 5	42.92	11.69	.086	PPMS	35.65	3.59	.491
	MS	n = 11	32.5	7.34		SPMS	32.38	10.55	
Lower Lumbar	Control	n = 5	49.92	1.41	.099	PPMS	40.54	13.6	.115
	MS	n = 6	37.60	17.26		SPMS	10.60	.....	

2.6.4 Spinal cords of SPMS have greater degree of atrophy than PPMS in the upper cord levels

When comparison of the CSA was performed between PPMS and SPMS, the mean CSA in SPMS was lower than CSA of PPMS in all segments; however, this difference was statistically significant only in the upper cervical segment. The upper cervical segment CSA of SPMS subjects was 28.5 mm^2, while the corresponding segment in PPMS showed a CSA of 35.8 mm^2 ($p = 0.022$) (Figure 2.3, Table 2.5).

SPMS has less GM and WM surface area compared to PPMS. Nevertheless, there was no statistical significant difference in the GM and WM surface areas between PPMS and SPMS. The only exception was the WM surface area of the upper cervical segments, which demonstrated significant difference between PPMS and SPMS ($p = 0.030$) (Table 2.6).

Effects of independent factors on the cross sectional area of the cord

In controls, the CSA of the cord was significantly reduced with age controlling for gender and cord level. Partial correlation between age and cord CSA showed a correlation coefficient of - 0.569 and a p value of 0.007. In MS, controlling for gender, cord level and type of the disease, there was no significant effect of disease duration ($p = 0.446$) or type of the disease ($p = 0.294$) on the cord CSA.

2.6.5 Lesions numbers and sizes

Number of lesions that have been identified was 76; average ≈ 2 lesions/section. There were 40 WM lesions, 26 mixed lesions and 10 GM lesions. Average lesions number per section was ≈ 2 lesions/section in the cervical, ≈ 2 lesions/section in the thoracic, and ≈ 1 lesion/section in the lumbar area. The mean lesion size was 7.18 ± SD = 8.8 mm^2 in the cervical, 1.70 ± 1.5 mm^2 in the thoracic and 2.59 ± 3.3 mm^2 in

the lumbar. According to lesions type, the mean lesion size was 0.8 ± 0.66 mm^2 in the GM, 2.3 ± 2.5 mm^2 in the WM, and 13.4 ± 9.6 mm^2 in mixed lesions.

When the two disease subtypes were compared, the mixed lesions sizes had a mean of 14.7 mm^2 in SPMS and 11.1 mm^2 in PPMS (p = 0.390), the pure WM lesions have a mean of 2.7 mm^2 in SPMS and 1.1 mm^2 in PPMS (p = 0.009), and pure GM lesions have a mean of 0.6 mm^2 in SPMS and 1.0 mm^2 in PPMS (p = 0.450).

Linear regression, controlling for age, gender, disease duration, disease type, lesion type, and cord level showed that the main predictor for lesion size was the lesion type, by being larger in mixed lesions (p < 0.001). When lesion type was dropped, the main predictor was the cord level by being larger in upper cord levels (p = 0.013).

Table 2.6: The CSA in the GM and the WM separately

level	Type of sample	GM		WM		Type of the disease	GM		WM	
		CSA (mm^2)	p	CSA (mm^2)	p		CSA (mm^2)	p	CSA (mm^2)	p
Upper Cervical	Control	7.63	.004	51.20	.000	PPMS	4.43	.197	31.32	.030
	MS	4.00		27.89		SPMS	3.62		24.88	
Lower Cervical	Control	13.03	.045	56.65	.008	PPMS	11.19	.269	44.17	.437
	MS	10.14		41.48		SPMS	9.80		40.58	
Thoracic	Control	2.64	.336	17.96	.186	PPMS	4.39	.060	27.04	.433
	MS	3.64		25.21		SPMS	3.18		24.12	
Upper Lumbar	Control	12.91	.049	30.01	.389	PPMS	6.26	.693	29.39	.108
	MS	6.73		27.43		SPMS	7.30		25.08	
Lower Lumbar	Control	17.66	.275	32.26	.056	PPMS	16.23	.126	24.31	.113
	MS	14.46		21.09		SPMS	5.65		4.95	

2.6.6 Proportion of demyelination

Proportion of demyelination is higher in the upper spinal cord segments

Demyelination affected mainly the cervical regions of the spinal cord (24%) compared to the thoracic (6.5%) and the lumbar regions (4.7%). Proportion of

demyelination was highest in the lower cervical region, where 26.8% of the cord was demyelinated, while the upper cervical cord showed 18.4% demyelination. In contrast, the upper lumbar showed 6.95% demyelination and the lower lumbar region showed only 0.58% of demyelination. Demyelination of the GM and the WM were observed to be greater in the higher cord levels (Tables 2.7 and 2.8).

Proportion of demyelination in SPMS and PPMS spinal cords

Regardless of cord level, overall demyelination was 8% in PPMS and 24% in SPMS ($p = 0.002$). In the upper cervical segment of PPMS degree of demyelination was 6%, compared to 29% in the corresponding area of SPMS ($p = 0.053$). In the lower cervical region the mean proportion of demyelination was 30.2% in SPMS and 16.6% in PPMS ($p = 0.247$). Similarly, results from the rest of the spinal cord segments also did not show significant differences between PPMS and SPMS in the degree of demyelination (Table 2.8, Figure 2.3).

Table 2.7: Proportion of demyelination in the main cord levels and in the main 5 segments categories.

Block level	Cord dem %		Segment Category	Cord dem %		Type of the disease	Cord dem %		P value
	Mean	Range (median)		Mean	SD		Mean	Range (median)	
Cervical	24.12	00-91(19)	Upper cervical	18.35	23.66	PPMS	5.93	00-28 (0.00)	.053
						SPMS	29.22	00-78 (28)	
			Lower cervical	26.83	28.34	PPMS	16.65	00-75 (0.6)	.247
						SPMS	30.22	00-91 (29)	
Thoracic	6.53	00-39 (0.9)	Thoracic	6.53	13.40	PPMS	2.09	00-6 (0.00)	.511
						SPMS	9.20	00-39 (2)	
lumbar	4.70	00-30 (0.00)	Upper lumbar	6.95	11.88	PPMS	7.50	00-26 (00)	.878
						SPMS	6.29	00-30 (00)	
			Lower lumbar	0.58	0.90	PPMS	0.70	00-2 (00)	.542
						SPMS	0.00	00-00 (00)	

Proportion of demyelination of the GM and the WM

By further categorising results by the area involved, the GM of upper cervical segment of PPMS was affected by 20% demyelination compared to 50% in the corresponding segment of SPMS ($p = 0.142$). The lower cervical cord GM was affected by 24% GM demyelination in PPMS and 34% GM demyelination in SPMS (p

= 0.476). Similarly, results from the rest of the cord segments did not reveal difference between the two forms.

Similar results were obtained from the WM demyelination, in which SPMS did not show difference from the PPMS. The only exception was the WM of the upper cord, which revealed significant difference between PPMS and SPMS (p = 0.043) (Table 2.8).

Proportion of GM demyelination is higher than proportion of WM

Proportion of GM demyelination was significantly higher than WM demyelination in all levels except lower lumbar cord (Table 2.8). Although the proportion of demyelination in the whole section and in the GM area was higher in SPMS, the ratio of GM demyelination to WM demyelination was higher in PPMS.

2.6.7 Effect of independent factors and correlation with disease duration

Studying effects of age, gender, disease duration, type of the disease, and cord level on proportion of demyelination showed that age of the patient (p = 0.001) and the cord level are significant predictors of proportion of demyelination. However, when we dropped age from the controlling factors, type of the diseasee (p = 0.014) and the cord level (p = 0.038) were significant predictors of demyelination. Disease duration showed a trend toward significance (p = 0.059). This points to the complicated relation between age of the patient and the disease duration.

Similar results were obtained when the previous regression model was applied on the WM demyelination. It showed that there is significant effect of disease type (p = 0.009) and cord level (p = 0.046). When the same regression model was applied on the GM exclusively, only the cord level showed significant effect on GM

demyelination (p = 0.049) with no effects of disease subtype on GM demyelination (p = 0.155).

2.6.8 Effect of demyelination on atrophy

Using Pearson coefficient, the correlation between degree of atrophy and demyelination was evaluated in the cervical cord. Bivariate correlation for the whole sample was -0.229 and p value was 0.122. However, considering the two subtypes of the disease and controlling for age, gender, disease duration, and post-mortem delay, there was significant correlation between proportion of demyelination and the cervical CSA in SPMS (correlation = -0.605, p = 0.001), but not in PPMS (correlation = - 0.557, p = 0.152).

Table 2.8: Proportion of demyelination in the 5 segments in the GM and the WM with the variation between PPMS and SPMS.

	GM dem %		WM dem %		Disease subtype	GM dem%		Sig	WM dem %		Sig	Dem ratio GM/WM
	Mean	Median	Mean	Median		Mean	Median		Mean	Media n		
Upper cervical	37.29	20.8	15.47	5.2	PPMS	20.00	0.0	.142	3.71	7.01	0.043	5.39
					SPMS	52.41	67.2		25.77	24.99		2.03
Lower cervical	31.25	17.85	25.98	20.1	PPMS	23.57	0.9	.476	15.10	24.61	0.212	1.56
					SPMS	33.81	28.7		29.61	28.80		1.14
Thoracic	9.00	0.00	6.23	1.0	PPMS	5.64	0.0	.638	1.61	2.79	0.496	3.50
					SPMS	11.02	0.0		9.00	17.00		1.22
Upper lumbar	17.36	0.00	4.95	0.0	PPMS	22.26	0.0	0.57	4.87	7.75	0.977	4.57
					SPMS	11.48	0.0		5.00	11.28		2.230
Lower lumbar	0.80	0.00	0.43	0.0	PPMS	0.96	0.0	0.57	0.51	0.75	0.568	1.88
					SPMS	0.00	0.0		0.00	...		0.0

2.6.9 Summary of results

In conclusion, decline in the CSA in MS cords compared to CSA of controls was statistically significant only in the upper and lower cervical segments. SPMS appeared to have a greater degree of cord atrophy than PPMS, but the difference was statistically significant only in the upper cervical cord. Reduction in the WM and

GM surface areas in MS compared to controls was significant in upper cervical and lower cervical segments.

Proportion of demyelination is higher in upper spinal cord levels and this applies to WM and GM demyelination. It seems that there is greater degree of demyelination in SPMS spinal cords, especially in the higher cord levels. The higher proportion of demyelination in SPMS affects mainly the WM of the upper cord levels. In addition, the mean size of pure WM lesions was greater in SPMS compared to PPMS.

GM demyelination was greater than WM demyelination in both disease forms, but the ratio of GM demyelination to WM demyelination was higher in PPMS than SPMS. In the present sample, neither atrophy nor demyelination correlated with disease duration in the whole sample, but the two parameters correlated with each other in SPMS subjects.

2.7 Discussion

The present study compared the cord atrophy and demyelination between PPMS and SPMS in multiple cord levels. It included a relatively large sample size, compared to previous MS histopathology studies [103, 105, 122]. Although there was a difference in the mean age of subjects between the two examined MS subtypes (PPMS 67 years and SPMS 58 years), the sample was controlled for the duration of the disease (approximately 25 years), scale of clinical disability [124], and for post-mortem delay (10 hours).

2.7.1 Comments on the possible effect of tissue shrinkage on interpretation of results

Since we have two sources of MS tissue, shrinkage factor may vary and may affect interpretation of results. However, tissue shrinkage is expected to affect the absolute values of the CSA and not the proportion of demyelination. A possible concern, which may lead the shrinkage to affect proportion of demyelination, is that the normally appearing areas may have different shrinkage factor from that of the chronically scarred tissue. This issue remains a possibility and needs to be investigated further.

To test the variability in the CSA, the CSA was calculated for the two sources of MS tissue and were compared together. Comparison included the exact segment level and not the segment category. The Pearson correlation coefficient was 0.877 and the p value was 0.010. Having strong and significant correlation in the surface areas of the exact cord level between two different sources of MS tissue supports our assumption that the expected atrophy from MS is presumably greater than effect of shrinkage (Table 2.4). In addition, the two sources of tissue have been differentiated in the graph in Figure 2.4. The graph shows that the two sources of MS tissue are located near each other compared to the location of controls form the same sources. Therefore, we were convinced that variation in shrinkage factor was significantly less than degree of atrophy and thus did not have considerable effects on interpretation of results.

2.7.2 Identifying the cord level

Classification of spinal cord segments into 5 categories is based on the normal enlargements of the spinal cord. A controlled morphologic study of human spinal cord was carried out by Kameyama in 2005 [147]. The human spinal cord has normally two enlargements, which are the cervical and lumbar enlargements. The

cervical enlargement occurs from C5 to C8 which is larger than upper cervical segment (C2 to C4) in the CSA and GM surface area. Similarly the lumbar cord has been classified into upper and lower segments where the lumbar enlargement mainly occurs at L4-L5. Therefore, L1, L2 and L3 were considered one segment and L4-5 as another segment (Figure 2.5).

Considering changes in morphology of the thoracic cord, there are no reliable morphological changes to differentiate between various thoracic segments [147]. In addition, there are no significant changes in the CSA between various thoracic segments (Figure 2.5). Therefore, the thoracic sections were classified as one segment. Other studies categorised the cord segments differently; for example, Gilmore et al (2005) and Evangelou et al (2005) performed pathological studies on MS atrophy and divided the thoracic cord into upper and lower thoracic segments. Due to the long thoracic segments, pathologies of the upper and the lower thoracic segments may vary [105, 112]. Although classification of the thoracic cord into upper and lower thoracic can be more useful, identification of the thoracic level depending on morphology is not as reliable as for the cervical and the lumbar cords.

2.7.3 Distribution of lesions

In this study, proportion of abnormal section within the cervical segment was 70%. MRI study of the spinal cord in MS showed that possibility of MS lesions in the spinal cord is 75% [123], which is more than our histopathology study. Our pathology study should presumably show more than 75% because histopathology studies are more accurate than MRI in detecting abnormalities [108]. This is probably because our sample does not include serial sections like MRI, and there is a chance that abnormal areas in higher or lower levels are missed.

Spinal cord lesions were commonly seen in the postero-lateral compartment [120]. The common postero-lateral lesions in MS may affect vital spinal cord structures such as the CST, PCP, and the DH. Symptoms related to lesions of these tracts are common in MS. For example, CST abnormalities in the brain were found to be associated with weakness in MS [148, 149]. Fatigue, which can affect up to 78% of MS patients [150], can be related to CST pathology, in that walking induces functional changes in the CST (measured by the motor evoked potentials) leading to central fatigue [151].

The significantly higher frequency of CST involvement in SPMS than PPMS may explain the suggestion that SPMS spinal cord pathology correlates better with disability [152]. The calculated higher proportion of demyelination and larger lesion sizes in SPMS in this study may be responsible for the higher frequency of lesions in the VH, DH, CST, and PCP in SPMS compared to PPMS.

The observation that some GM lesions are limited by the GM/WM boundary and take the shape of the VH may be due to physical limitation caused by two different environments at the WM/GM border or may imply that the GM pathology is different from the WM. The isolated midline lesion of the PC was frequent in our sample and has been reported previously [120]. Such lesions can be due to mechanical pressure or secondary to axons' degeneration in the midline within fasciculus gracillis. These midline axons come from the lower limbs and have a long course in the CNS, and are thus more liable to lesions.

2.7.4 Predilection for the cervical cord

Demyelination and atrophy appeared to have predilection for the cervical spinal cord in the two disease forms. There is obvious preservation of the lower cord levels. In

addition, we found that SPMS has a higher degree of demyelination compared to PPMS, which was more obvious in the upper cord. There was a trend toward significance between the two disease forms in the upper cervical cord when the proportion of demyelination of the whole cord was examined ($p = 0.053$). Similarly, SPMS demonstrated more atrophy than PPMS, which was only significant in the upper cervical segment.

Significant atrophy in the higher cord levels, with less atrophy of the lumbar regions, has been mentioned in other studies [105, 127]. Although the calculated higher CSA in the thoracic cord of MS subjects may not be reliable in the present study, because we have only one thoracic block as a control, upper thoracic segment distension in MS has been observed previously with much bigger control sample size [112].

Gilmore et al suggested that preservation of the lower cord CSA is due to lesser amounts of WM compared to GM. The assumption was based on observation that GM is well preserved in the cord. In this study and another two previous studies, GM atrophy of the spinal cord was observed [124, 127].

Rarity of lesions in the lower cord may play a role in preservation of the lower spinal cord from atrophy. In progressive MS spinal cord lesions detected by MRI constitute only 5% of newly-detected lesions [138]. We calculated that the average lesion per section was 2 lesions/section in the cervical level and 1 lesion/section in the lumbar level. The mean lesion size was 7 mm^2 in the cervical cord and 2 mm^2 in the lumbar cord ($p = 0.003$). However, the relationship between demyelination and atrophy is not well understood. From the data above, it seems that there is agreement between atrophy and demyelination of the cord in that both of them have predilection to affect the cervical cord. Previous studies that have examined for correlation between

demyelination and atrophy in the spinal cord reported different results. An MRI study showed that atrophy of the cord correlates with degree of demyelination [115]. In the present study, there was significant correlation between lesion load and atrophy in SPMS only. Evangelou and his colleagues studied local effects of spinal cord lesions on the degree of atrophy in 55 MS subjects and 33 controls. There was no correlation between demyelination and atrophy. They attributed this finding to atrophy of the normal-appearing areas. This does not conflict with the results of this study, because there was no correlation between atrophy and demyelination in the whole sample. The correlation was found only in SPMS.

However, it is not clear why the lower cord is a rare site for MS lesions. On presentation, site preference has been detected in PPMS and SPMS. Early in the disease course spinal cord lesions are more related to PPMS, with a few small lesions in the brain [153], while SPMS is more associated with brain lesions. It is possible that both forms have predilection to affect higher levels of the CNS in the early stages. Early in the disease process there is significant demyelination and atrophy of the spinal cord in patients with less than 5 years' disease duration [111]. In fact, the spatial progression of CNS atrophy in the rostro-caudal direction over time has been reported in the brain. The fact that atrophy of MS is confined to supratentorial structures in the early disease course and then progresses to infra-tentorial structure later on may partly explain the relative sparing of the caudal levels of CNS [152].

2.7.5 Variation between PPMS and SPMS

Several studies have compared degree of demyelination between PPMS and SPMS in the brain and the spinal cord, including cross sectional and longitudinal studies. Most of those studies showed a higher degree of demyelination in the SPMS spinal

cords, but not all of them reported significant difference. It was found that lesions affecting PPMS patients appear to be fewer and smaller [153]. PPMS has been found to be associated with an MRI picture of diffuse abnormalities in the brain and the spinal cord, rather than focal lesions. There was a higher degree of demyelination in the brains of SPMS, with no difference at the spinal cord level [102]. Kidd and colleagues demonstrated a greater degree of demyelination in SPMS spinal cords, which did not reach statistical significance [138]. A study by Thompson showed that the difference in the proportion of demyelination between PPMS and SPMS does not include the spinal cord region but is restricted to the brain [139]. Alternatively, in 1999, Stevenson conducted MRI study of the brain and the spinal cord, and showed that degree of demyelination was higher in the brains and spinal cords of SPMS [154].

Variation among studies seems to occur between cross sectional studies [102, 103, 153, 155, 156], while longitudinal studies consistently show more frequency of new lesions in SPMS [138, 139, 157]. This can be due to difference in the baseline disease duration among studies. Rate of atrophy and occurrence of lesions are not constant throughout the disease course. The preceding relapsing phase of SPMS is associated with larger lesions and with profound atrophy of the brain and the spinal cord compared to PPMS [102]. Rate of atrophy in the first 10 years (3.5% per year) was greater than rate of atrophy during the entire course of MS (0.75% per year) [112]. But with progression, frequency of new spinal cord lesions is quite rare in the two disease forms and PPMS was associated with an even higher rate of spinal cord atrophy compared to SPMS [157].

Therefore it is possible that causes of atrophy in early MS are different from causes of atrophy in late MS; subsequent slow atrophy in progressive MS may be due to

degenerative process rather than direct effect of the disease, especially in PPMS. For example, spinal cord atrophy in progressive MS was found to be significant over one year of follow up without detecting new lesions [138, 158]. Over 5 years of follow up a correlation was found between atrophy and clinical deterioration, but not between atrophy and lesions [159].

While effects of disease duration on the CSA of the cord has been demonstrated to be significant previously [105, 112, 114, 152], there was no significant correlation between duration of disease and CSA of the cord in the present study. We further examined the relationship between the cervical-segment CSA and the disease duration. The graph shows that MS patients who died within the first 10 years of having the disease have the highest rate of decline in the cervical CSA. This raises the importance of tissue atrophy in morbidity and mortality in MS patients. Although this is not a longitudinal study, the graph in Figure 2.6 may indicate that rate of atrophy is high in the first 10 years of the disease. Although there is a normal decline in the volume of the spinal cord with age, MS subjects showed reduced volume of the spinal cord compared to the age matched healthy controls.

2.8 Conclusion

The spinal cord is affected by demyelination and atrophy in progressive MS. This is more obvious in the cervical cord. There seems to be a greater degree of demyelination and atrophy in the cervical cord of SPMS subjects compared to PPMS subjects. The size of WM lesions appears to be greater in SPMS, but PPMS shows a higher tendency to affect the GM.

Atrophy of the spinal cord is significant in MS and does not appear to follow a steady rate over time. Atrophy seems to be caused by multiple factors. Effects of these

factors on atrophy are not steady through the disease course, resulting in a variation in the rate of atrophy over time. Initially, in the first 5 years of age, there is high rate of atrophy as shown by previous studies. With more progression of the disease duration, rate of atrophy becomes slower and may reach a plateau. This can be concluded from the graph in Figure 2.6. The slow rate of atrophy after a few years of the disease is possibly caused by degeneration of small axons and other elements, and not by demyelination. This is because frequency of new spinal cord lesions is very low.

We believe that SPMS causes more spinal cord demyelination and atrophy because it causes more frequent lesions and more inflammation, which possibly causes more tissue scarring. This may explain the significant correlation between atrophy and demyelination in SPMS and not in PPMS.

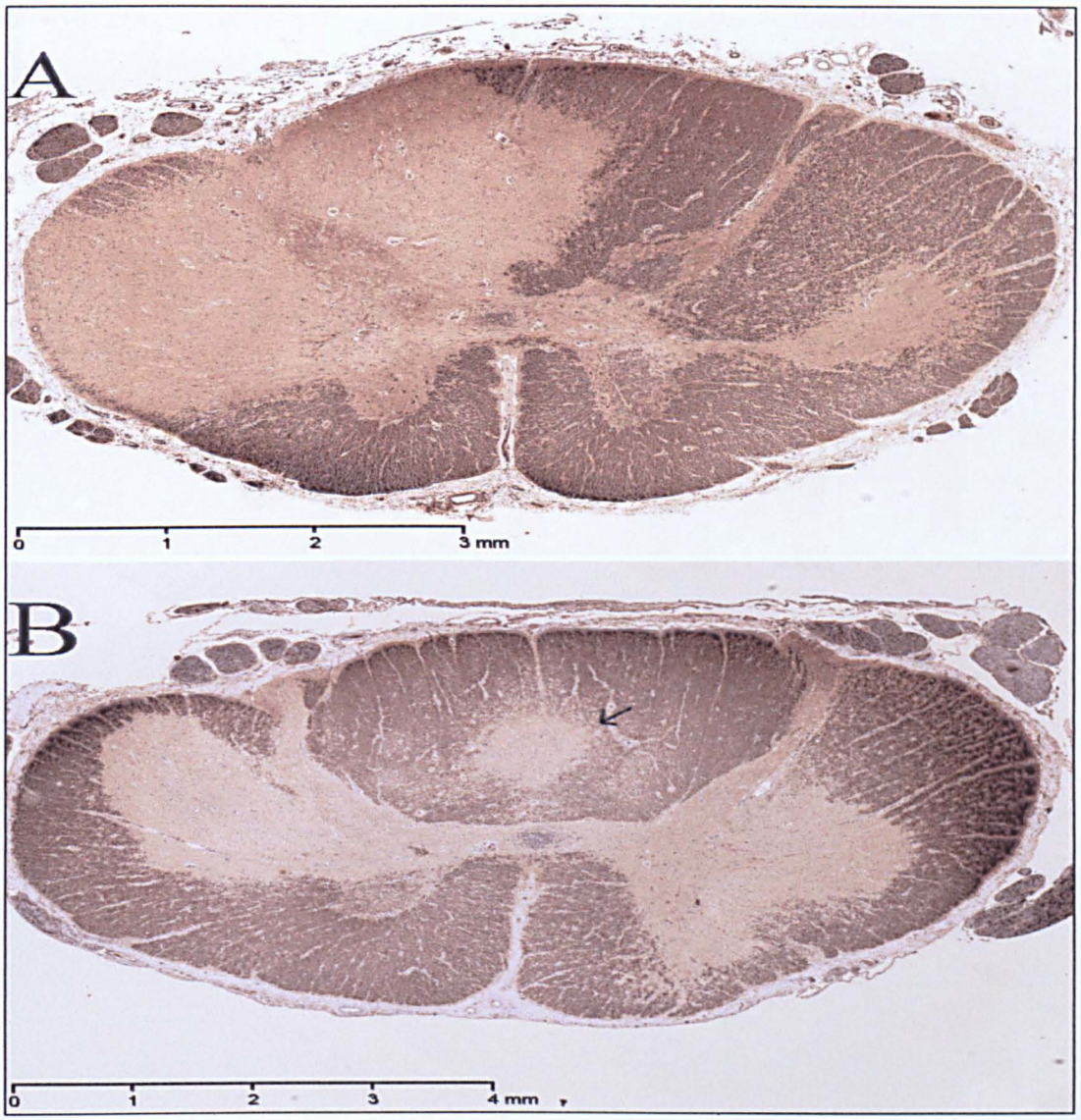


Figure 2.1: MBP stain of spinal cord section showing lesions affecting the right side of the cord in A (left side of observer). Note that the lesion involves the WM and the GM but also respect the boundary of the VHs in both sides and did not pass to the adjacent WM. Demyelination extends from the right side to affect the left side through the intermediate GM without passing to the nearby WM (in A and B). These two observations were commonly seen. (The scale bar = 3 mm). The arrow in B points to a commonly lesion of the PCP. The lesion is frequently symmetrical and bilateral (The scale bar = 4 mm).

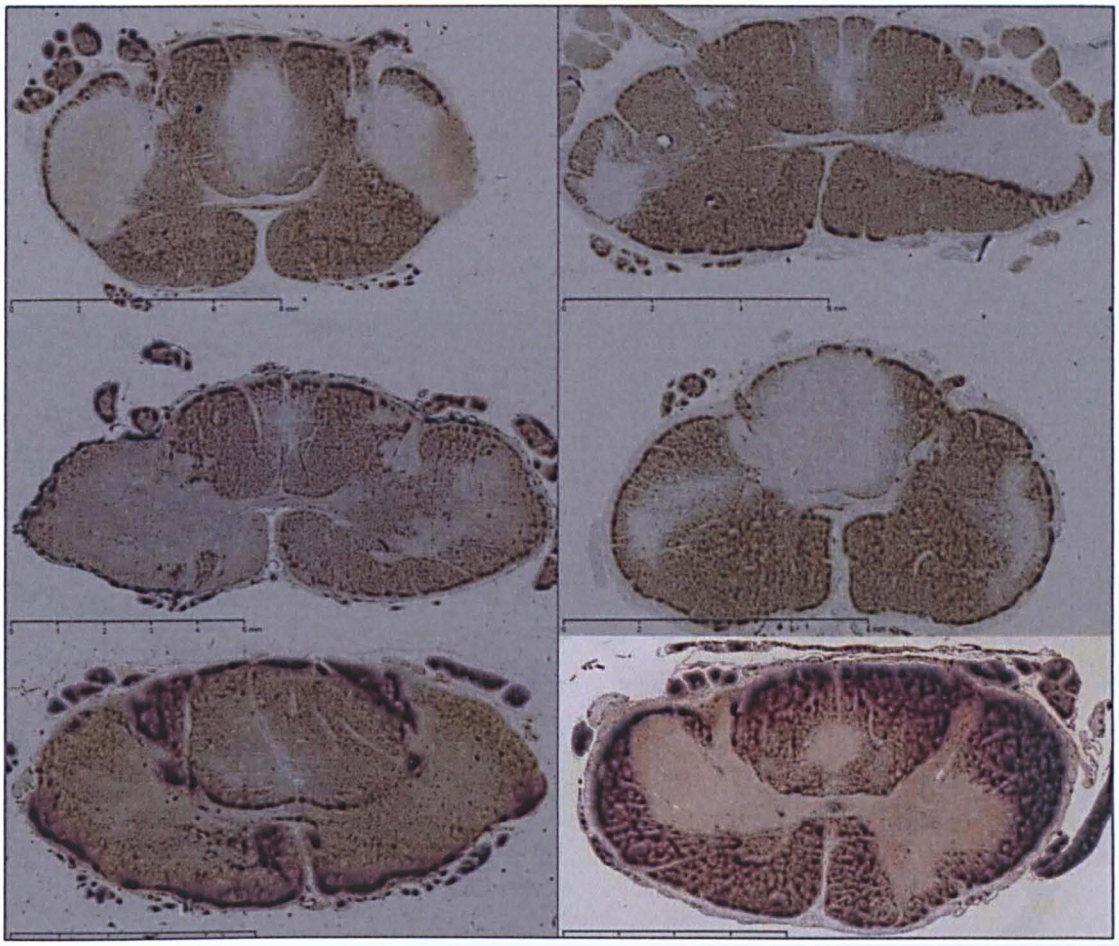


Figure 2.2: Six examples of the common PCP lesion. The lesion is usually midline, symmetrical and bilateral. Size of the lesions varies greatly. It is frequently associated with bilateral lesions of the lateral funiculus.

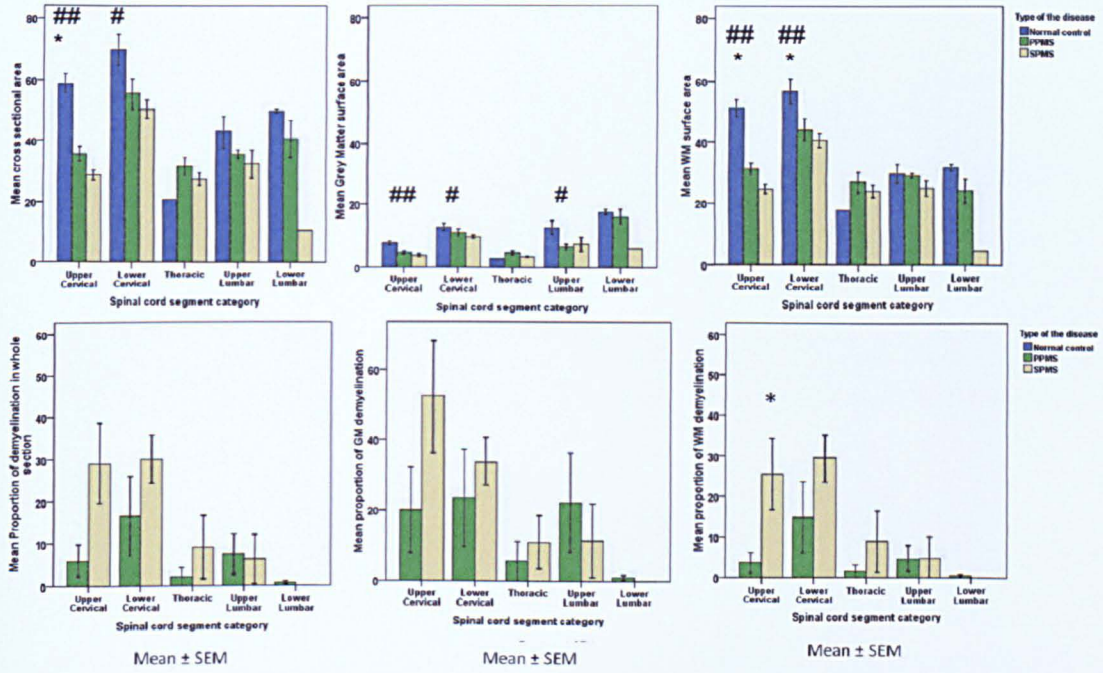


Figure 2.3: Bar charts showing the variation in the CSA and proportion of demyelination in various spinal cord levels in controls, PPMS, and SPMS. Note the highest proportion of demyelination is found in the GM, which may be due to its small size. The data represent the mean CSA ± SEM and the mean proportion of demyelination ± SEM. The significant difference between PPMS and SPMS is indicated by an asterisk, while the significant difference between MS and controls is indicated by the number sign #. The p values < 0.05 are indicated by one sign, while the p values < 0.01 are indicated by two signs.

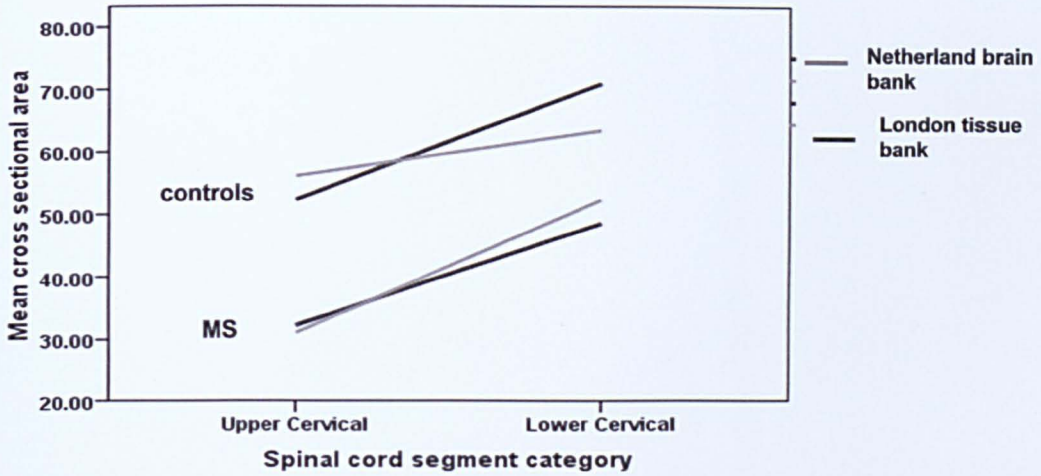


Figure 2.4: The graph demonstrates the CSA of the upper and lower cervical cord in MS and controls in the two sources of tissue. Only the cervical cord included because for the rest of the cord, we have one source of MS tissue. Note source of tissue and the consequent variation in shrinkage between the two sources of tissue appear to have little effect on the CSA compared to atrophy caused by MS.

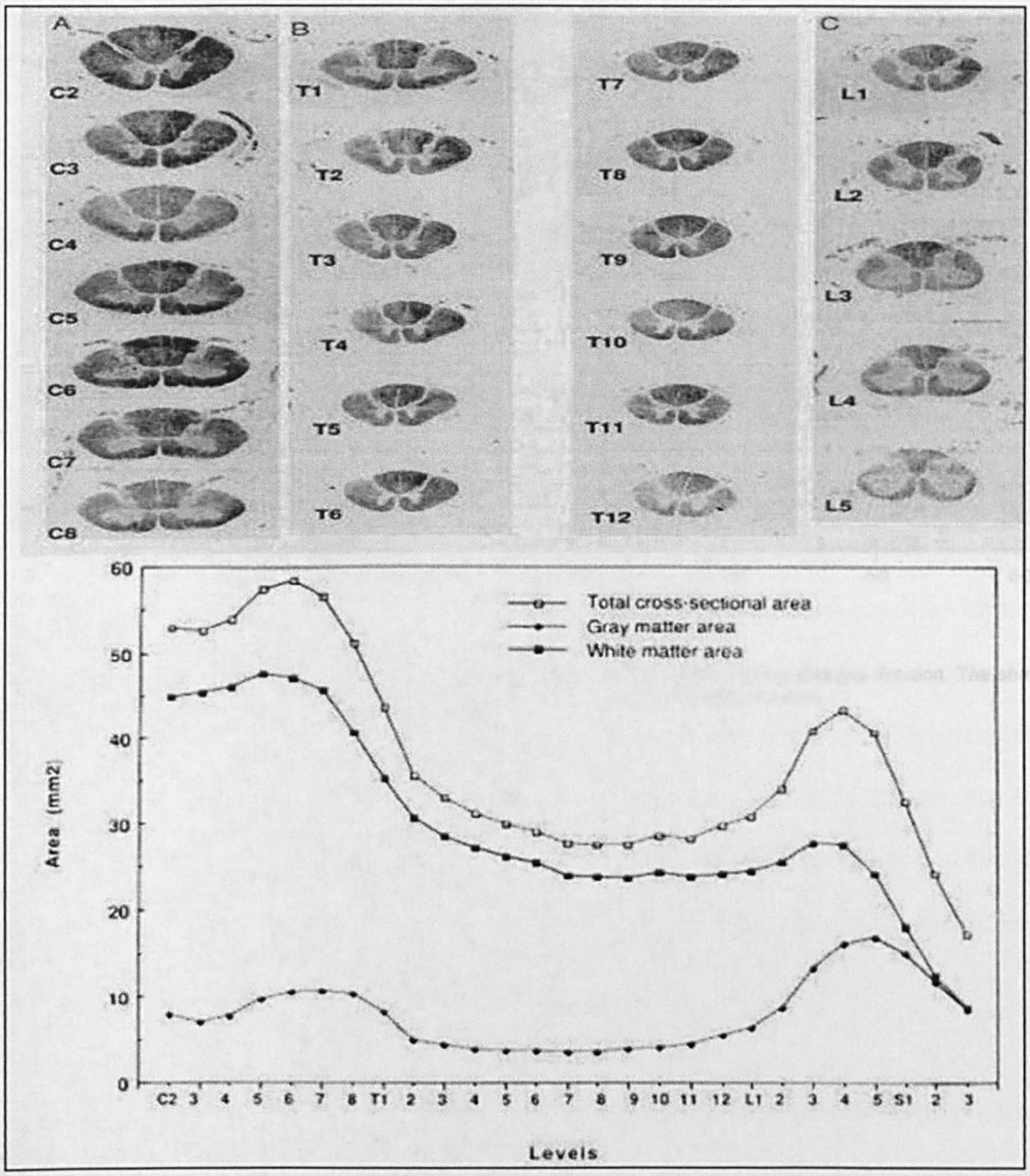


Figure 2.5: The CSA in mm^2 of the spinal cord segments from C2 to S3. Note the cervical thickening and lumbar thickening, which provides the basis of categorizing the spinal cord into 5 segments; upper cervical, lower cervical, thoracic, upper lumbar, and lower lumbar [147].

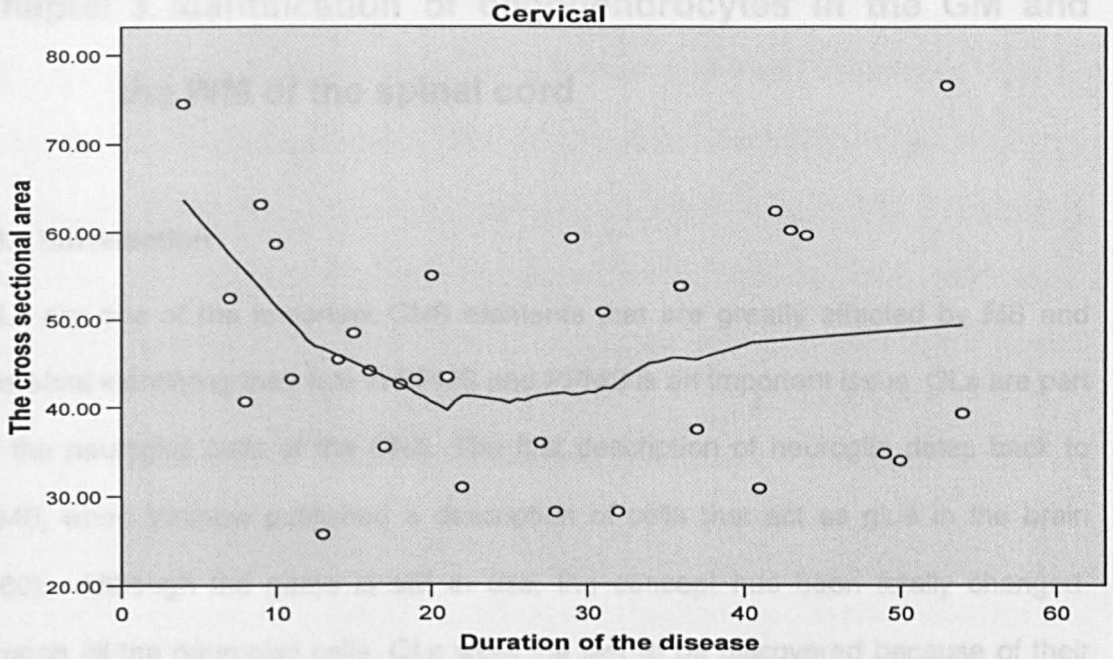


Figure 2.6: The CSA of the cervical cords in our study sample and their relation to the disease duration. The sharp decline in the CSA in the cervical cord in MS is seen in the first 10 years of disease duration.

Chapter 3: Identification of oligodendrocytes in the GM and the WM of the spinal cord

3.1 Introduction

OLs are one of the important CNS elements that are greatly affected by MS and therefore identifying their fate in SPMS and PPMS is an important issue. OLs are part of the neuroglial cells of the CNS. The first description of neuroglia dates back to 1846, when Virchow published a description of cells that act as glue in the brain [160]. Although the name is still in use, the concept has been totally changed. Among all the neuroglial cells, OLs were the last to be discovered because of their high resistance to staining agents [161] and their small size (8-15 μm) [162]. The term OLs was introduced for the first time in 1921 by Rio-Hortega when he used the metallic impregnation method to visualise the neuroglia. He initially discovered the astrocytes by using the gold impregnation, then he used the silver carbonate impregnation to visualise the OLs and microglia. Metallic impregnation stains both the cell body and the processes [160].

In addition to staining difficulties, there are other difficulties with quantification of OLs; OLs are sensitive and affected widely by changes in environment during culture and by the stains during slide preparation; OLs form extensive networks of branches in three dimensional space with very complex connections [163]. It has been suggested also that the number of OLs is not constant during stages of maturation and their number is reduced with age progression [164], health status [165], and by chronic use of alcohol or anti-psychotic drugs [166].

3.1.1 Structural features and classification of oligodendrocytes

Although the name implies that OLs have few processes, these cells form an extensive network of fine branches [162]. OLs have variable morphologies depending on their location in the CNS and the stage of maturation (Figure 3.1). According to distribution in the CNS, Hortega recognised three types of OLs; near axons (interfascicular), near cell bodies (perineuronal), and near vessels (perivascular) [167].

White matter (interfascicular) oligodendrocytes

According to the size and interaction with other cells, Hortega classified WM OLs into four subtypes. These subtypes are distributed variably within the CNS, and each type myelinates certain diameter and number of axons. Type I is found in the forebrain and cerebellum and myelinates up to 50 small axons (15-20 μm), while type II is located in different sites of the WM and myelinates fewer and thicker axons (20-40 μm). Type III, which myelinates only 3-4 larger axons, is more distributed in cerebellar peduncles, medulla oblongata, and the spinal cord. The rare type IV OLs have no branches and are restricted to the exit of the nerve root areas. Each type IV OL myelinates only one large diameter axon and greatly resembles Schwann cells [167].

The cytoplasmic density of OLs is variable under the electron microscope by being dark (electron dense) for the mature cells and lighter for the less mature cells (Figure 3.1). The cytoplasmic density reflects the amount of ribosomes and degree of chromatin condensation. There are not many differences among OLs subtypes in types of organelles inside the cell apart from their densities [160, 162, 168, 169].

These subtypes were recently found to be expressing different molecules. The S-isoform of myelin associated glycoprotein (MAG) is expressed in types III and IV and not in types I and II [170]. There is also evidence that these subtypes have variable expression of MBP and proteo-lipid protein (PLP). It was found that small fibre myelin has more MBP content, while large diameter fibre myelin has more PLP content [171]. Nevertheless, OLs still share common features that differentiate them from other cells.

OLs are small cells with large round or oval nucleus that is almost always eccentrically located leaving a large mass of cytoplasm at one pole of the cell. The nuclear membrane is rimmed by chromatin that extends into less dense nucleoplasm. The endoplasmic reticulum is well developed with large amounts of ribosomes. OLs appear moderately electron dense compared to astrocytes. According to the type of organelles inside the cells, there are two differences between astrocytes and OLs; OLs have neither glycogen granules nor bundles of specific intermediate filaments, such as GFAP which is a characteristic of astrocyte [160, 167, 169, 172].

Grey matter (perineuronal or satellite) oligodendrocytes

The term perineuronal OLs was also used for the first time by Rio-Hortega in 1921 [162, 167]. This term was used for OLs that are located in close proximity to neurons of the GM. The exact function of these perineuronal cells is not well known. It has been suggested that these cells have functions related to structural and functional support to neurons [160, 162, 167].

Ludwin described the ultrastructure of GM satellite OLs in a study in 1984. His description has not many differences from WM OLs. Satellite GM OLs were seen to

be oval with clumped chromatin. They were seen to be abutting and extending processes to numerous myelinated axons with the formation of the inner and outer lope of mesaxons [172, 173]. Cragg 1976 also classified perineuronal GM OLs with respect to their cytoplasmic density into light, medium, and dark OLs (Figure 3.1) [169]

3.1.2 Identification of oligodendrocytes

The optimal target for counting is to identify all OLs without extensively staining their processes. It is difficult to recognize OLs by light microscope using a classical stain such as haematoxylin and eosin. This is due to the difficulty in differentiating different types of glia. Although astrocytes are generally larger in size and have lighter cytoplasm, some light OLs may resemble astrocytes in size and cytoplasmic density [174].

During development of OLs, different proteins are either down regulated or up regulated according to the stage of differentiation without apparent change in morphology [175]. Therefore, the only way to distinguish between differentiating OLs is by detecting these markers [160]. It is possible to evaluate the number and density of OLs by means of immunohistochemistry. Immunohistochemistry utilises several antigens to identify OLs, such as MBP, PLP, MAG, cyclic nucleotide phosphodiesterase (CNPase), carbonic anhydrase II (CA II), galactosylceramide (GalC) and others. Immunohistochemistry also provides information about the cell identity as well as the functional state of the cell. These markers have variable distribution within the OLs in that some of these markers are traced in the cell body and the processes, while others are only present in the cell body.

Most of these markers can be also traced by in situ hybridization of their mRNA. The mRNA localisation can reach as far as the Ranvier node for some proteins such as PLP or CNPase. This is because free ribosomes can reach as far as the site of myelin synthesis, due to the action of microtubules [176, 177]. In this pilot study two markers, which have been used to quantify OLs, will be compared; these are CA II [174, 178] and CNPase [165, 179]. CA II has been also used to stain and trace GM OLs in spinal cords (animal study) [180].

Carbonic anhydrase II

CA II mainly stains the cell body [174, 176, 180]. CA II catalyses the hydration of carbon dioxide to bicarbonate. In the CNS, CA II can be found in two forms; soluble form (55%) and membrane bound form (45%) [181]. CA is found in seven isozymes (from CA I to CA VII) [181], which are distributed in different types of mammalian tissues with variable amounts [176]. In humans there are two major iso-enzymes; CA I and CA II, which differ in both enzymatic activity and in antigenic properties (antibody that recognises CA II does not recognise CA I) [176].

The enzyme is vital for the function of erythrocytes in removal of carbon dioxide. CA II may also play a role in K+ homeostasis [176] and lipid metabolism [181]. Other functions of CA may include regulation of pH, water balance, gluconeogenesis, bone resorbtion, and formation of CSF. CA inhibitors are already in use as anti-glaucoma drugs, diuretics, and anticonvulsive drugs [182]. Deficiency in CA II has been reported in humans and found to be associated with mental retardation, brain calcification, osteopetrosis, and renal tubular acidosis [183].

CA II expression in OLs has been identified in humans between 16 and 22 weeks of gestation. CA II is expressed in the cell bodies and processes during early

differentiation of OLs and it is found in all stages of OLs development [184], but in adult myelinating OLs it is restricted only to the cell body. However, expression of CA II is reduced with age in both OLs and choroid plexus, which might explain the reduction on CSF production in old age [182].

CA II is considered as a specific marker for both mature and less mature OLs. This has been demonstrated by double labelling of OLs by CA II and MBP [185]. Tracing CA II by means of immunohistochemistry has been stated to be a good method for counting OLs [174], as it stains only the cell body. It was suggested that CA II is found in astrocytes , choroid plexus, some neurons, especially GABAergic neurons [182, 186]. later it was possible to recognise that these are actually immature OLs, which are frequently mistaken for astrocytes or small neurons [185].

CA II enzyme has been appearing in literature since 1932 [187]. Around 4865 articles that have carbonic anhydrase in their title can be found on the Pub med and around 787 articles on the subject of CA II (last accessed on 23 Aug 2010).

Dr Ghandour, who published 19 studies that utilized the CA II in OLs, stated via personal communication that immunohistochemistry with CA II stains all types of OLs in human CNS, and that both GM and WM show good results with paraffin-embedded tissue sections. A number of studies have described the immunohistochemistry protocols for CA II tracing in OLs in animals [188, 189] and in humans [182].

2′3′-cyclic nucleotide 3′-phosphodiesterase

2′3′-cyclic nucleotide 3′-phosphodiesterase, or CNPase, is a marker of mature and less mature OLs [165]. It accounts for 4% of the myelin protein. It hydrolyses 2′3′-

cyclic nucleotides [184], but the physiological role of this interaction is not well understood. However, patients with Down's syndrome and Alzheimer's disease have low levels of CNPase [164].

CNPase consists of two polypeptides; CNP1 and CNP2. CNPase appears early during OLs differentiation and is synthesized in the cell body, but deposited near the plasma membrane and in myelin sheaths [190].

CNPase antibody is capable of reacting with mature and immature OLs, and thus can stain most OLs. It reacts in both human and rat with high penetration of thin sections [165]. This enzyme is specific for OLs and not expressed in astrocytes or other cells [164, 165].

A number of studies have utilised CNPase immunoreactivity to count OLs in humans [165, 177, 179] and in MS specifically [179]. In 1999 Dr. Claudia Lucchinetti studied quantitatively OLs in MS plaques. The study utilised a number of antibodies such as MOGs, CNPase, and MBP antibodies [179]. Claudia Lucchinetti has also stated via personal communication that CNPase is an excellent marker for labelling OLs.

In another study human autopsies were used to compare numbers of OLs in the cerebral cortex of schizophrenic patients with normal controls. CNPase antibodies have been used to trace and count OLs in the cerebral cortex. CNPase antibody was considered as a robust marker for mature and nearly mature OLs. CNPase monoclonal antibody detects OLs specifically and distinguishes them from astrocytes and other elements [165].

It has been suggested that CNPase tracing does not stain all GM OLs [177]. CNPase labelling was used to evaluate the effects of ischaemia on human cortical OLs following cardio-respiratory arrest. CNPase positive OLs were clustered in layers IV, V, VI of the cerebral cortex, with few CNPase positive cells in the outer laminae. 71% of all satellite OLs have been found positive for CNPase [177].

3.2 Aims and hypothesis

OLs are important in MS disease and quantifying them efficiently is essential. Our main aim from this study is to develop a reliable and efficient counting method for all OLs lineage in both the GM and the WM. In the literature different markers were used to quantify OLs. CA II and CNPase have been explicitly said to be ideal for counting OLs [162, 165, 185]. CA II labelling can be more helpful for counting OLs, as it stains only the cell body [162]. CNPase was reported to be a robust marker and ideal for counting and tracing mature and nearly mature OLs [165]. Therefore, we compared CA II and CNPase and studied the abilities of both markers to identify OLs and showed the anatomical structures of the spinal cord such as the GM, the WM, and the DH laminae.

3.3 Material and methods

3.3.1 Autopsy sample

Two blocks were selected randomly from the MS tissue sample. These are human spinal cord autopsies from the MS Society tissue bank in London. The process of initial fixation of the tissue from the MS Society tissue bank was mentioned in Section 2.5.1. Briefly, the frozen blocks were defrosted and formalin-fixed paraffin-embedded blocks were prepared. Then 15 sections were cut from each block at 5 μm thickness to optimise the immunohistochemistry of the two markers.

3.3.2 Immunohistochemistry of oligodendrocytes

CNPase mouse monoclonal antibody and CA II rabbit polyclonal antibody were purchased from abcam [191, 192]. Optimisation was carried out by an expert technician, Neil Hand. The slides resulted from each dilution were reviewed by Prof Jim Lowe, Consultant Neuropathologist. The best results for CNPase were obtained with pre-treatment using 10 mmol sodium citrated at pH 6 with a dilution of 1:2000. This was heated in a microwave oven. The CAII sections were pre-treated with 10 mmol EDTA at pH 8. For antigen retrieval, sections were heated in microwave oven for 25 minutes. The primary dilution was 1:8000 for 1 hour. This was detected using Rabbit/Mouse Envision (Dako K5007) for 30 minutes. All sections in both stains were counterstained with haematoxylin. All sections were stained automatically using Tech Mate 500+ immunostainer (details in appendix B).

3.3.3 Slides scanning

Slides were numbered and scanned by the NDP view Hamamatsu at magnification of 40X. The produced digital slides were then viewed by the NDP.view software and demarcations were performed using the manual graphic tablet MD 85637 from Medion. Features of the machine and the associated software can be found in Section 2.5.3 and Hamamatsu web site [144].

3.3.4 Comparing CA II and CNPase

General observations

The appearance of the GM and the WM and the ability of the stain to show abnormally demyelinated areas were observed in the two stains. The GM boundary and DH laminae were identified and demarcated as mentioned previously (Section

2.5.4). The ability of the stain to show OLs as distinguishable cells from the surrounding neuropil was considered at high magnification.

Sampling the GM and the WM

We sampled the VH and the area of the lateral funiculus (lateral CST) to represent the GM and the WM respectively. From each section, the VH and the CST were sampled using a strict protocol which allows the examiner to select the same region from the two sections (Figure 3.2). The sampling protocols of the VH and the CST are mentioned in details in subsequent chapters (see Figures 4.1 and 5.1). The regions of interest were exported as an image of the computer screen when the magnification is 40X. These regions were exported as JPEG format images and were given codes that do not refer to the type of the stain. In total, we calculated OLs in 34 fields from the VH (17 CNPase and 17 CA II) and 40 fields from CST (20 CNPase and 20 CA II).

3.3.5 Counting process of oligodendrocytes

OLs were counted in each field so that the observer was blinded to the type of stain (it is difficult to identify type of stain at high magnification in a small field; 0.047 mm^2). The exported images were viewed by imageJ software. ImageJ is popular software for image processing. ImageJ is freely available image editor software that has multiple functions such as editing, processing and performing automatic and manual counting of particles. The software can also measure surface areas and pixel values. The freeware of ImageJ can be downloaded from the internet [193].

OLs were counted manually and categorised into possible OLs and definite OLs. The identification criteria of OLs include intensely stained cells, round to oval cell bodies of variable size. However, very small rounded dots were excluded from counting in

both markers because they represent myelinated axons. Cells with light staining, stained rim, or irregular shape were all considered possible OLs.

Using imageJ, the field was opened and the manual counting was carried out. With the computer mouse cursor, all typically stained OLs were selected. The software automatically counts the selected points. Then the colour of selection is changed and the possible OLs were then selected. The software, again, counts the new particles and put them in a different group. Therefore, two numbers were produced from each counting process; one representing definite OLs and the other possible OLs. The ratio of possible OLs to definite OLs was calculated for both CA II and CNPase. Reliability of our identification criteria and reproducibility of our counting process has been tested by repeating OLs counting by two observers (interobserver reproducibility was 16%).

3.3.6 Scoring system

We developed a scoring system for comparing the two stains that depends mainly on microscopic observations and quantification measures. Factors which have been taken into account at low magnification include the ability of the stain to demarcate GM various structures and the ability to obscure demyelinated and partially myelinated areas. At higher magnification, the ability of the stain to show OLs clearly and the calculated ratio of possible OLs to definite OLs were included in the ranking system.

3.3.7 Statistics

Results were exported to SPSS 14 software. The data table contained field code, number of definite OLs, number of possible OLs, and ratio of possible to definite OLs. Student t-test was used to compare the means.

3.4 Results

3.4.1 Observations at low magnification

Both CA II and CNPase can demarcate the GM from the WM fairly well. Some CA II sections had less clear borders of GM making delineation with the WM harder. In fact, in CA II the intensity of the GM stain is more than the WM while in CNPase the reverse is true. The border between the GM and the WM in CNPase is clear and resembles that seen in MBP stain (Figure 3.3).

The identification of spinal cord major laminae was possible using both stains to the same degree. The unmyelinated substantia gelatinosa (Laminae II and III) is well demarcated and greatly helps in differentiating the marginal zone (lamina I) dorsally and the nucleus proprius (Laminae IV and V) ventrally. Both CNPase and CA II are able to distinguish the GM and different laminae of the DH to the same degree (Figure 3.4).

An interesting observation is that demyelinated areas are not detectable in CA II-stained sections. Ability of the CA II to obscure areas of demyelination and partial myelination is useful in increasing the observer blinding to demyelinated areas. This is not the case with CNPase stain, which shows abnormally myelinated area and thus may reduce the examiner blinding when counting OLs, especially when manual counting is to be carried out. Another remarkable observation is that the GM and its boundary are frequently identifiable in CA II-stained sections even with the presence of severe demyelination (Figure 3.5).

3.4.2 Observations at high magnification

In CA II and CNPase, OLs appeared generally heterogeneous in size with round to oval shape. The OLs processes and cytoplasm could not be identified in most of the

sections (Figure 3.6). There were frequent counterstained cells with traces of CNPase or CA II stain around their border, which was more obvious in CNPase-stained sections.

Because of the staining of the nearby myelin with CNPase, OLs do not appear as clear as in CA II-stained sections, especially in the WM as it contains more myelinated axons [176, 194, 195]. The CA II shows less intensity in the myelin sheath than in OLs. Therefore, staining myelin with CA II did not interfere with OLs counting and showed OLs clearly. Non specific staining of counterstained cells was minimal in CA II.

3.4.3 Numbers of oligodendrocytes

The mean density of definite OLs varied between the two stains. The mean density of definite OLs in the GM of CNPase-stained sections was 48.98/mm^2 compared to 167.28/mm^2 in the CA II-stained sections ($p = 0.006$). In the WM, the CA II-stained sections showed also significantly more density of definite OLs than CNPase stained sections (119/mm^2 vs. 31/mm^2, $p < 0.001$). On the other hand, the mean density of possible OLs in the GM of CNPase-stained sections was 27.40/mm^2 and in CA II-stained sections was 5.78/mm^2 ($p = 0.05$). In the WM, the calculated density of possible OLs in CNPase-stained sections was 33.17/mm^2 and in CA II-stained sections was 19.66/mm^2 (p value = 0.265).

The mean ratio of possible OLs to definite OLs was 0.6 in the GM of CNPase-stained sections compared to 0.03 in CAII-stained sections. In the WM the ratio was 1.1 in the sections stained with CNPase and 0.2 in sections stained with CA II.

3.4.4 The scoring system

The scoring system for this study favours the use of CA II, as it seems to be superior to CNPase in the identification of OLs and in obscuring areas of demyelination or partial myelination. But both markers identified the GM and the laminae of the DH to the same degree (Table 3.1).

Table 3.1: Comparison between CNPase and CA II

Factors	CA II	CNPase
Ratio of query OLs to definite OLs	1	0
Identifying the GM	1	1
Identifying the DH various laminae	1	1
Obscuring the demyelinated and partially myelinated areas	1	0
Identification of OLs in an isolated form	1	0
	5	2

3.5 Discussion

This study is probably a pioneer study in quantification of OLs in the spinal cord particularly in the GM, thus our aim is to quantify all OLs regardless of maturation status. There are a number of markers that can be traced in OLs during maturation. It is important to differentiate between two types of OLs markers; maturing OLs markers and OLs/myelin markers. For example, markers such as nestin, platelet derived growth factor α receptor (PDGF-α), ganglioside GD3, NG2 proteoglycan and the O4 are all markers for maturing OLs and are not myelin markers [160].

Nestin is a class of intermediate filaments that are found in neuroepithelial cells and some radial glia. Expression of nestin is downregulated in differentiated OLs [196]. PDGFR-α is expressed in the early stages of OLs lineage and not in mature OLs. Ganglioside GD3 is not very specific for OLs in vivo. Its expression disappears as the OL matures. It is considered ideal for identifying OLs in culture [197]. NG2 marker is an integral membrane proteoglycan. These NG2 positive cells may also express PDGFR-α in the early stages. However, these cells continue as a distinct population in the adult CNS and do not express markers of mature OLs (for review of NG2 positive cells, see Nishiyama et al (1999)) [198]. The O4 monoclonal antibody marks

unidentified glycolipid in OLs. It labels a specific stage of OLs maturation called pre-OLs, or sometimes called pro-OLs, and does not cover all maturation stages [199].

Markers of OLs and myelin include GalC, RIB antigen, CA II, NI-35/250 proteins, and myelin specific markers (CNPase, PLP, MBP, MOG, MAG, and other minor proteins). GalCs are early markers of OLs. It is located on the surface of early OLs and continues to be expressed on the surface of mature OLs [200]. It is believed that they appear to express in the late stages of pro-OLs. However, labelling GalC in the early stages of OLs maturation is inconsistent, due to lack of specificity of the used antibodies [160]. MOG is a glycoprotein that is expressed in latest stages of OLs myelination [201]. The rest of the myelin specific proteins such as MBP, PLP and MAG are markers of myelinating mature OLs and thus do not cover all maturation stages. Therefore, we compared CA II and CNPase because CA II expression covers all the stages of OLs maturation and continue to be expressed in mature OLs [202] and CNPase is the earliest marker that can be reliably labelled in OLs [203, 204].

The method used allows for comparison between the two markers in the GM and the WM in the same anatomical regions. To accomplish this, a sampling protocol was used to select fields from the GM and the WM. In conclusion, the results obtained from this pilot study favour the use of CA II stain over CNPase for counting OLs.

The CNPase stains OLs and the nearby neuropil. Although it forms 4% of the isolated myelin proteins, CNPase is not present in the compact myelin. It is localised in the cytoplasm of non compacted myelin sheaths and the cytoplasm of OLs processes [205-207]. CNPase appears early during development of OLs and continues to be expressed in OLs and myelin [160]. It is synthesised primarily in the cell body and then transferred to be deposited in the myelin sheath. This explains the

presence of the stains in the surrounding neuropil and myelin. In comparison, CA II minimally stains the neuropil. The ability of the CA II to obscure demyelinated and partially myelinated areas in most of the cases is an important feature of this marker. At low or high power no demyelinated or partially myelinated areas could be identified with CA II (Figure 3.5). At high power, however, only the lesion border can be identified on CA II stain.

Another important feature is the ability of the CA II stain to delineate the GM borders within the lesion. In the spinal cord MS lesions extend from the WM to the GM without respecting GM/WM boundary. The MS lesions usually appear homogenous without clearly showing the GM and the WM architecture in a number of stains, such as MBP, H&E, and with CNPase. But with CA II it was possible to demarcate the GM from the WM in sections, even when there is MS lesion. This was obvious when you compare the MBP and CA II stains in Figure 3.5.

The capability of the two markers to differentiate between different laminae of the DH was noticed to be similar. Applying the sampling protocol on the whole DH may include some areas from the normally unmyelinated substantia gelatinosa, which is found in the middle of the laminae between the marginal zone (lamina I) and the nucleus proprius (Lamina IV and V). Therefore, choosing the nucleus proprius to represent the DH was essential for number of reasons; first, the nucleus proprius is directly related to an important function which is the proprioception; second, the nucleus proprius is heavily myelinated and contains numerous OLs; and finally the nucleus proprius is large enough to be sampled.

At high magnification, OLs were recognisable using the two markers. Features of OLs, such as oval to round cells with variable sizes, were recognisable in the two

stains. Although OLs were variable in sizes and they tend to be small cells, the misinterpretation of OLs as axons was not possible. Large myelinated axons were identified by having central faint, while OLs were homogenously stained. Nevertheless, OLs appeared clearer with CA II marker.

In the scoring system, we included the ratio of possible OLs to definite OLs. Comparing the two markers by counting only the definite OLs number across the spinal cord can be an unreliable process, since each marker has different sensitivity in various areas. It was mentioned previously that both CA II and CNPase do not stain all OLs, because CA II is not present in certain types of OLs [175], while CNPase stains about 70% of GM OLs [177].

Counting the possible OLs that appeared in the fields and comparing them may also not be a reliable process, since each marker may have a different degree of non-specific staining of other cells. We believe that possible OLs are OLs that do not have the same intensity of staining as other OLs. Therefore, the ratio of possible OLs to definite OLs was considered in the ranking system rather than the absolute numbers. From this study one can conclude that the ratio is more with CNPase than CA II. This means that CA II stains more definite OLs. The sum of possible and definite OLs was also greater in CA II-stained sections, which may indicate that CA II stains more OLs. In fact the absolute number of definite OLs was greater in CA II-stained sections. Most importantly, histopathologic appearance of the two stains has been reviewed by Prof Jim Lowe (Consultant Neuropathologist) who found that with CA II stain, recognising OLs was better.

For many reasons, these results could not be generalised. Firstly, this is only a pilot study with a limited number of cases. Secondly, 74 fields from only two subjects were

analysed and this may not be representative. Thirdly, the sample includes MS cases' autopsies which were dissected from the cadaver at variable times after death and thus different proteins may denature faster than others. This means that if CNPase denature faster than CA II, a comparison at fixed post-mortem delay period will result in more OLs with CA II. Finally, apart from the first criterion, other criteria of the scoring system are subjective and depend on the research goals. For instance, the criterion of obscuring demyelinated areas may not be desirable for other types of study that do not require unbiased selections and therefore CNPase marker would be favoured over CA II in this case. Furthermore, CA II probably stains more OLs because it covers all stages of OLs lineage, while CNPase stains only mature and nearly mature OLs [202].

All the criteria included in the ranking system are directed toward establishing a reliable and reproducible quantification process for OLs, and our decision depends on our research objectives. Therefore, a marker such as the CNPase is still considered a robust marker for labelling OLs.

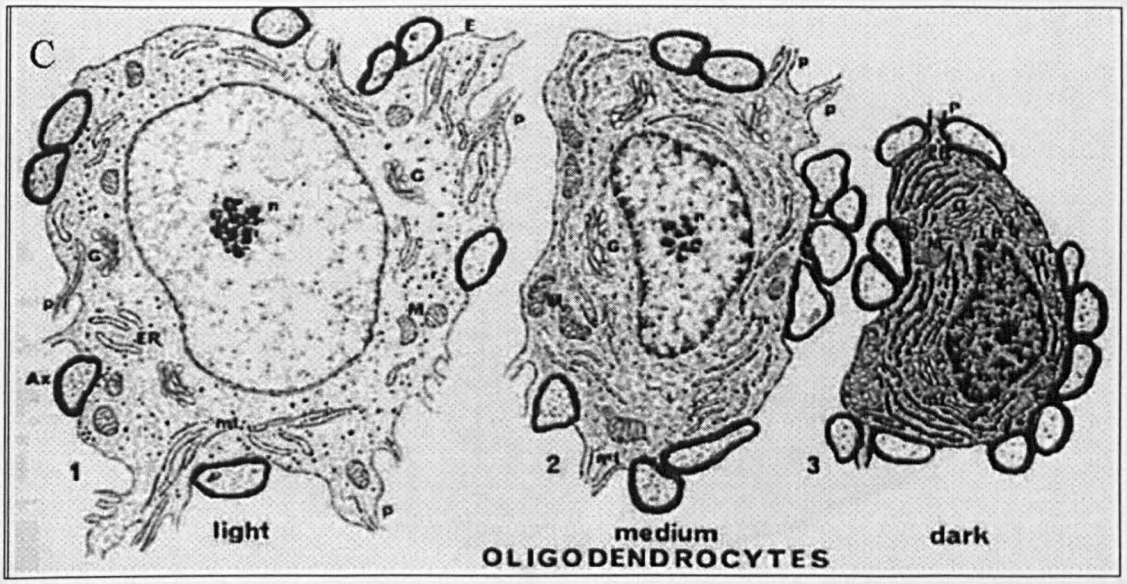


Figure 3.1: Illustration of the three subtypes of OLs as seen under the electron microscope in rats [208].

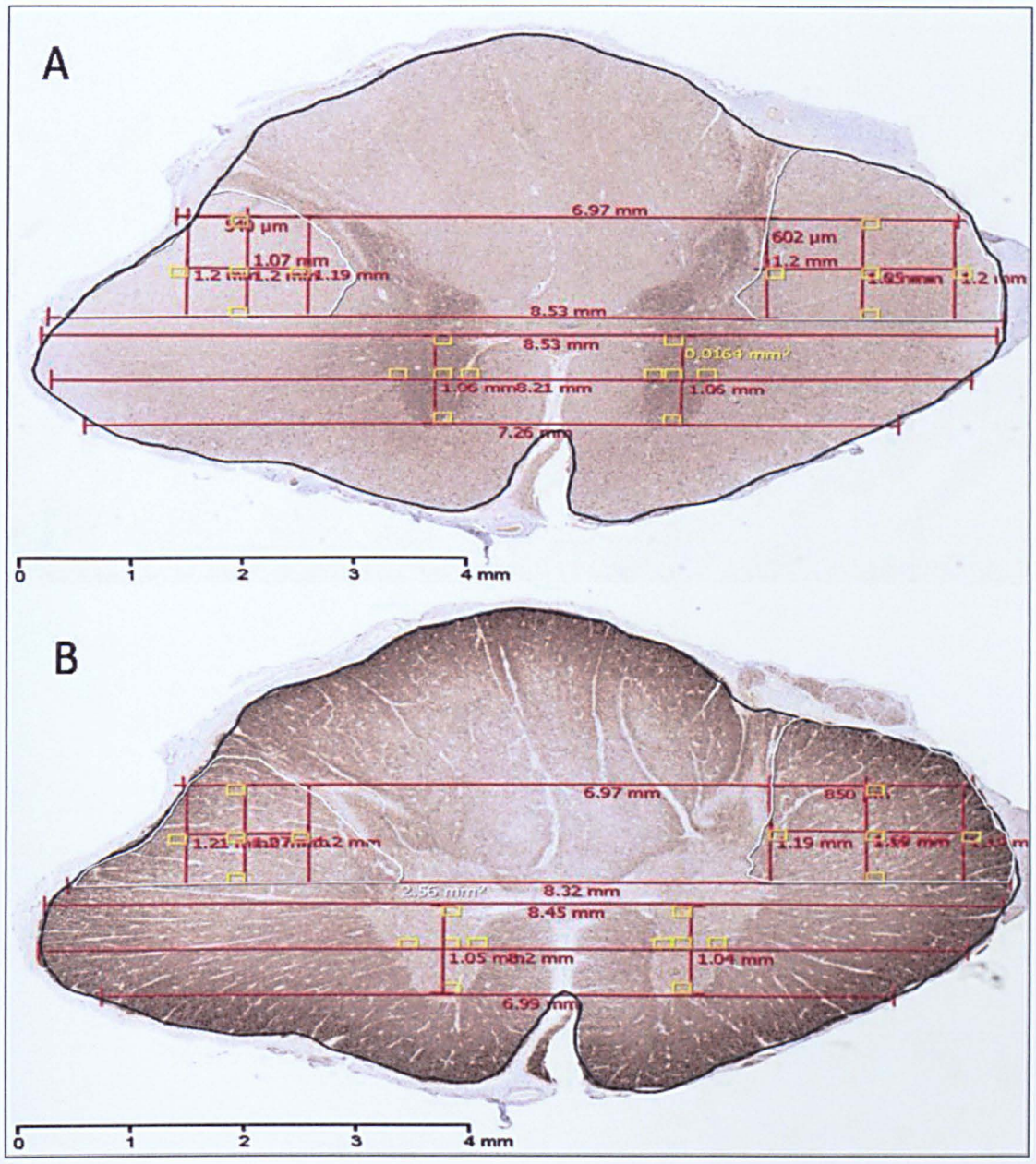


Figure 3.2: Two differently stained sections that belong to the same subject and same spinal cord level (CA II-stained section in A and CNPase-stained section in B). Note the extensive staining of myelin in B. The sections were sampled using strict protocol that allows the investigator to compare fields taken from the same anatomical area. Details of sampling protocols of the VH and the CST are mentioned in chapters 4 and 5. The area within the white border is the area occupied by the CST (scale bar 4 mm).

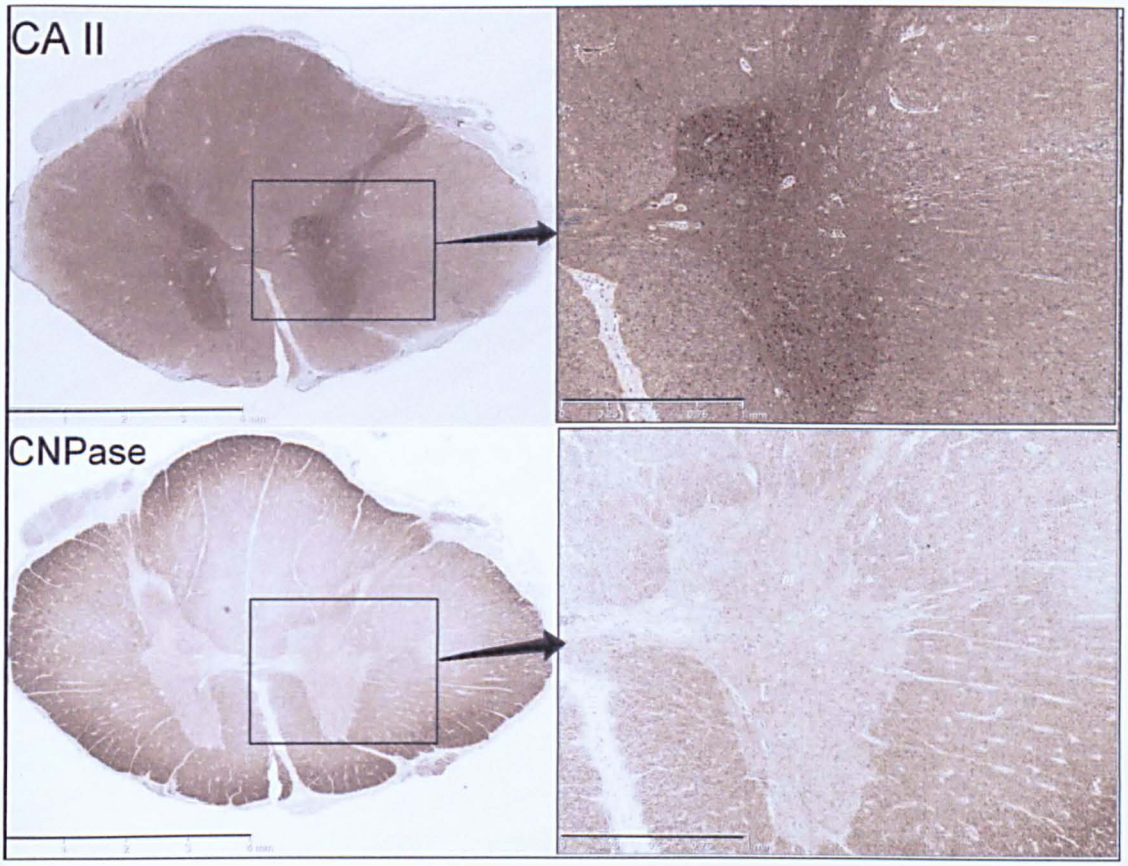


Figure 3.3: CA II and the CNPase at low magnification (scale bar 4mm). Note the sharp demarcation of the GM in CNPase compared to CAII at higher magnification (scale bar 1 mm).

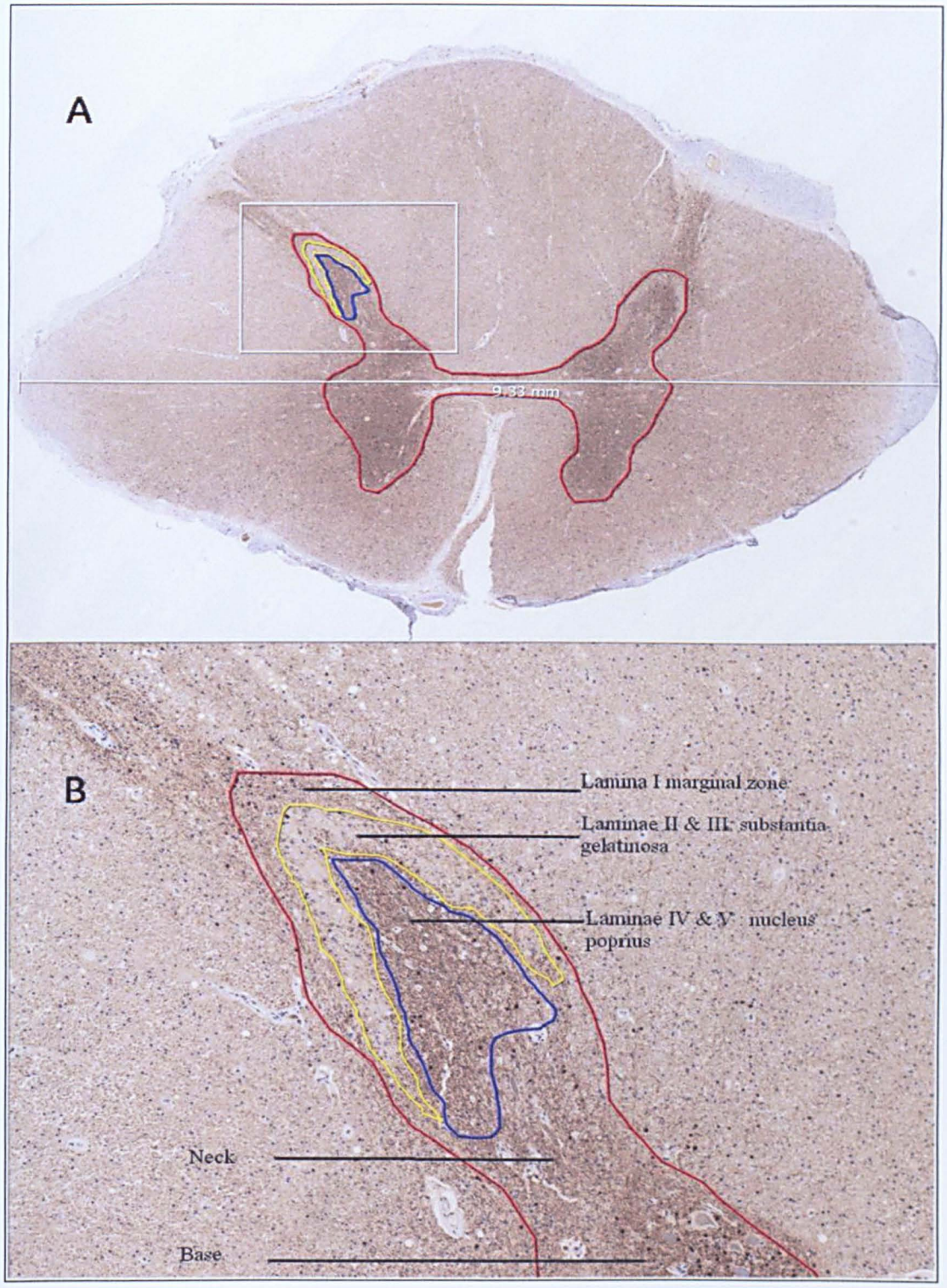


Figure 3.4: In A, CA II stained spinal cord cross section. We demarcated the GM and the DH major structures such as the nucleus proprius within the blue border, the substantia gelatinosa within the yellow border. The rectangle is magnified in B. The cap of the DH is a thin layer forming Lamina I. The head of the DH is formed of the substantia gelatinosa Laminae (Laminae II and III) and the nucleus proprius (Laminae IV and V). The neck is continuous with the base and both of them form Lamina VI.

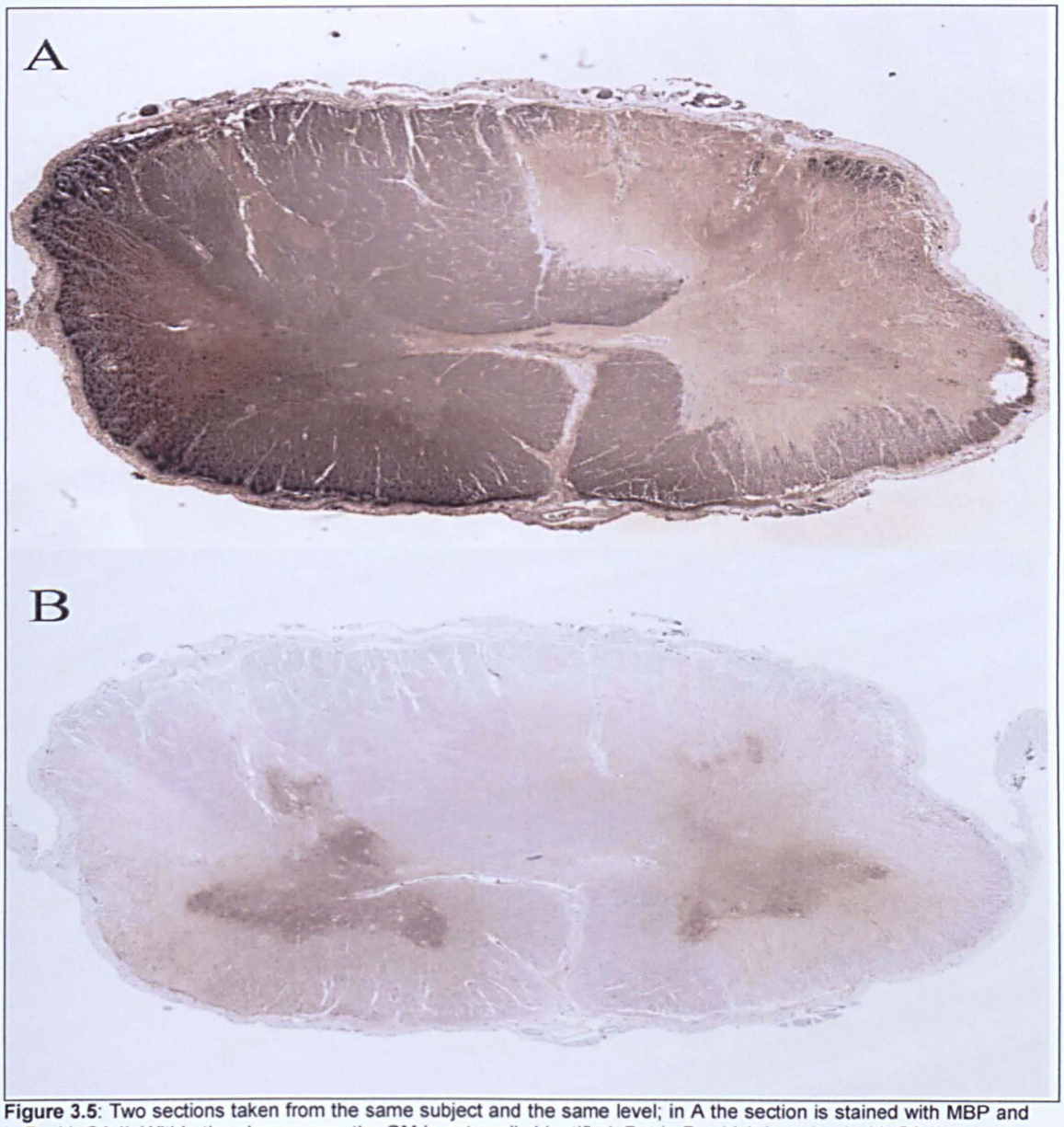


Figure 3.5: Two sections taken from the same subject and the same level; in A the section is stained with MBP and in B with CA II. Within the plaque area, the GM is not easily identified. But in B, which is stained with CA II stain, the plaque does not appear while the GM boundary is prominent

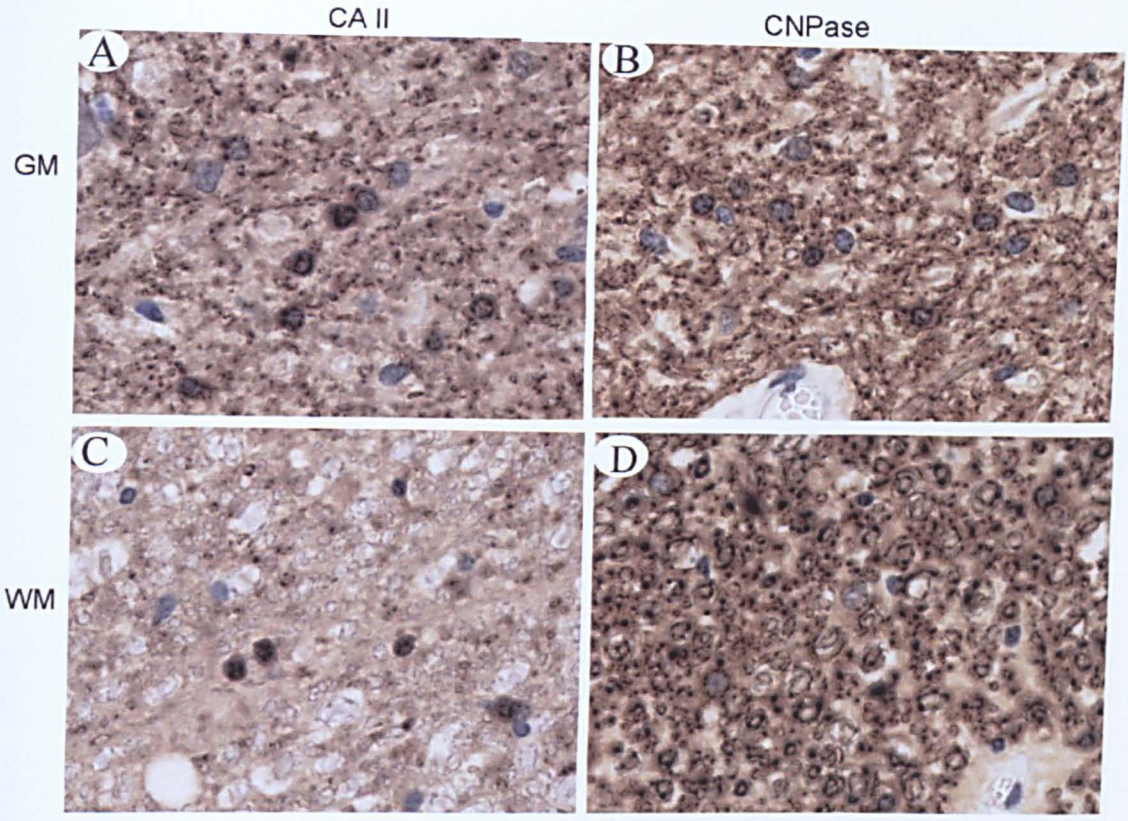


Figure 3.6: OLs as they appeared at high magnification power in CA II (A&C) and CNPase (B&D). A & B fields compare the GM in between the two stains, while B & D fields compare the WM. Note in both the GM and the WM; myelin is less stained in CA II sections making OLs easily identifiable. It can be noticed also that in CNPase myelin is more intensely stained in the WM compared to the GM.

Chapter 4: Pathology of oligodendrocytes in the GM of progressive MS spinal cords

4.1 Introduction

The spinal cord GM has myelinated axons that traverse the GM and WM boundary. These axons are myelinated by the widely-dispersed OLs in the WM and the GM [174]. OLs are possibly vulnerable to injury in the GM following MS. Involvement of the GM in MS was reported in the literature as early as 1898 [121], while morphological description of the spinal cord GM lesions was described by Fog in 1965 [209], Oppenheimer in 1987 and recently by Gilmore in 2009 [105, 120]. In the WM, injury to OLs usually results in demyelination of axons and reduction in the speed of the action potential. This has been demonstrated in animals [210] and in humans [211]. In comparison, consequences of OLs loss in the GM are uncertain.

A number of observations support that GM OLs are different from the WM OLs. For example, many GM OLs acquire a perineuronal position and may react to MS differently. OLs in the GM may have additional functions such as providing trophic support to neurons. Moreover, spinal cord GM has different stages of development and final architecture from the WM and from the brain GM [212]. In MS specifically, there are number of observations that suggest that GM pathology is independent of WM pathology; two previous studies demonstrated that spinal cord GM demyelination is more extensive than WM demyelination [105, 127], which was also shown in the study of proportion of demyelination in chapter 2 of this thesis. Bo et al (2007) demonstrated that subpial cortical demyelination is independent of WM demyelination [213]. Degree of inflammation and recruitment of inflammatory and cytotoxic cells also varies between the GM and the WM of the brain; GM lesions has significantly less apparent inflammation [103, 214]. Variation between GM and WM

pathology in MS extends to include degree of remyelination; Albert et al 2007 conducted post-mortem study on 33 patients with chronic MS and reported that GM remyelination is more extensive than WM remyelination [215]. Therefore, it may be essential to study the fate of GM OLs in MS separately from WM OLs.

The majority of previous studies on GM pathology in MS, both in vivo and in vitro studies, were carried out on the brain GM (for reviews refer to Bo 2006 [121], and Geurts et al 2008 [216]). In spite of the increasing significance of GM pathology, there is no previous study that has specifically investigated the fate of OLs in the GM.

Due to the lack of information about the fate of GM OLs in MS, the rest of the introduction will discuss normal development of OLs in the CNS in general and the spinal cord in particular. The introduction will also review all stages of development until maturation of OLs and production of myelin. This will provide a preface to the next chapter, which studies WM OLs. The previously published work that investigated pathology of OLs in WM MS lesions is reviewed in the next chapter.

Studying normal development of OLs has enabled researchers to suggest and develop potential therapeutic strategies for MS. These strategies were developed from molecules that share functions with different OLs mitogens and have been proven to be effective in EAE [217-220]. For example, a new treatment strategy was derived from a sphingolipid, sphingosine-1-phosphate, that affects differentiation and migration of OLs [221]. Phase III clinical trials of fingolimod (FTY720) in MS are showing promising results [222]. Although fingolimod acts as immunomodulator and may not have effects on OLs, it was originally derived from the signalling molecule sphingosine-1-phosphate (most recent review by Coelho et al 2009 [218]).

In addition, understanding OLs' development may explain and consequently resolve two important issues; why GM in MS demonstrated more extensive remyelination than the WM [215], and why remyelination fails with disease progression in both GM and WM.

4.1.1 Development of spinal cord oligodendrocytes

The spinal cord and the brain develop from the neural tube [212]. The neural tube is formed from ectoderm by a process called neurulation, where a closed tube is formed from the neural plate. When the process of neurulation starts, a single layer of cells can be found lining the tube. This single layer is formed of a homogenous population of precursors called the neuroepithelial stem cells. These non-branching cells are multipotent and they can give rise to both neurons and glia. The cellular layer of the tube starts to proliferate to a multicellular layer [212].

The process of development of the spinal cord GM is different from that of the cerebral cortex. Rostrally, the tube starts to dilate forming the brain vesicles, while caudally, the tube does not dilate. Therefore, neuronal cells do not migrate a very long distance, but stay around the tiny central canal forming the spinal cord GM. In the brain neurons and neuroglia migrate longer distances from the ventricular zone to the cerebral cortex [223].

The last cells that are generated from the sub-ventricular zone are the OLs [224]. OLs' progenitors (OPCs), which are positive for the PDGFR-α, proliferate in the ventral part of the spinal cord subventricular zone [223]. Therefore, this study will also examine if there is variation in OLs' density between the ventral and the dorsal regions of the cord.

OPCs migrate and distribute widely in the WM and GM. The processes of proliferation and then migration are affected by several trophic factors and biological molecules [223]. One of the most important stimulating factors for migration is the PDGF. PDGF is considered as the major mitogen for OLs [223, 225]. PDGF acts on the PDGFR-α found on the surface of OPCs, which is expressed on the ventral neuroepithelial stem cells [225]. OPCs originate in the ventral region around the embryonic day 14 [194, 225] and they express, in addition to PDGF-α, the NG2 chondroitin sulphate proteoglycan [194, 226], O4, and DM-20 (the gene encoding for PLP subunit) [194]. At this level of development OPCs do not express markers of mature OLs, such as MBP or CNPase [194].

The significance of PDGF in stimulating OLs' proliferation, and subsequently remyelination, has been tested on animal studies. Allamargot at al (2001) examined acute demyelination of the corpus callosum in Wistar rats by injecting intrathecal PDGF. Allamargot and colleagues reported 49% increase in OLs and 60% reduction of demyelination within 2 weeks. Remyelination to demyelination ratio has also increased by 10 fold. Complete remyelination was noted in some animals within 3 months with no evidence of significant gliosis [219]. Effects of PDGF also extend to include chronic demyelination. In 2007 Vana and colleagues examined chronic demyelination of the corpus callosum in mice. They reported significant effects of PDGF on proliferation and regeneration of new OLs [220].

Another important factor which induces proliferation is a chemokine known as CXCL1 [227]. CXCL1 stimulates proliferation of OPCs and acts in synergism with PDGF in inducing migration by acting on specific receptors (CXCR2) on the surface of the spinal cord OPCs [227]. In MS, CXCL1 is probably produced from activated astrocytes [217]. Inducing EAE in mice that were treated to produce high amounts of

CXCL1 resulted in significantly less inflammation and demyelination. There was also a high numbers of OLs associated with extensive remyelination [217].

An important factor associated with OLs' lineage is insulin-like growth factor, which induces migration and accumulation of OPCs post-injury and thus stimulates remyelination [228, 229]. Expression of this protein was found to be extensive at the lesion border of demyelination in animals [230] and in MS [231]. However, at the lesion border in MS, there was also extensive expression of the insulin-like growth factor binding proteins. These binding proteins are believed to be potent inhibitors of myelination [231].

After this, migration of OLs is inhibited and cells start to proliferate again under the effect of PDGF and CXCL1. At this point in development and around birth, immature OLs become multi-processed and immobile but maintain expression of PDGF and NG2 [194]. Most of these OLs are still immature and thus not myelinating [160, 232, 233]. This explains the duration of extensive myelination between postnatal day 10 and postnatal day 60 as revealed by MRI studies [234].

As development continues, OLs lose expression of PDGF and NG2 (except for a population of cells that continue to express NG2 during adult life, these cells are called adult OPCs) and enter a stage of intermediate pro-OLs, which is the O4+ stage [194]. These O4+ cells are more mature, less motile and do not migrate [227]. On the other hand, NG2 positive cells continue as a distinct population of cells and spread in the GM and WM during adult life. The fate of NG2 cells and their role in oligodendrogenesis is discussed later in the thesis.

Initially, an excess number of pro-OLs is produced from proliferation of PDGFR-α positive cells and distributed widely in the GM and WM. Nevertheless, the programmed cell-death process (apoptosis) controls the final number of OLs so that it matches myelination requirements [194, 223, 227]. The final stages of maturation include expression of mature OLs' markers, such as PLP, MBP, MOG, and CNPase. As these cells mature, they start myelinating axons.

In summary, to reach the appropriate number of axons and the appropriate amount of myelination, the progenitor cells migrate, proliferate, differentiate, and then undergo timely programmed cell death [227]. The ultimate cytoarchitecture of OLs in the spinal cord involves interfascicular OLs and perineuronal OLs. Interfascicular OLs are heterogeneous in morphology [235] and in biochemical composition [175]. The process of establishing normal spinal cord cytoarchitecture depends largely on the correct localisation of the OLs and axons [223].

We have emphasized that OLs are a heterogeneous population of cells and each type myelinate a different number of axons [167, 174, 175]. However, the process by which these myelinating OLs acquire their distinct morphology is not clear. Early in development, PDGFR-α positive cells begin migration in response to other factors which act as guidance to these OLs [223]. These factors may include electric field currents, mechanical factors, or chemotactic substances, but it is not well understood whether these cells are programmed to a specific morphology or they acquire this morphology after migration and under the effects of the new environment. Studies on cultured neuroepithelial stem cells showed that all stem cells cultured under the effect of PDGF give rise to OLs [225]. This is an important issue in MS and has been raised in a number of MS studies to explain why OLs in chronic lesions fail to mature and gain the functional and morphologic characteristics of mature cells [236-239].

4.1.2 Functions of oligodendrocytes

The only proven function of OLs is myelination of the CNS axons, which is ascribed mainly to interfascicular OLs, while perineuronal OLs are assumed to have functions other than myelination, such as supporting and protecting neurons [169, 240, 241]. Intercellular junctions, including gap junctions and to a lesser extent tight junctions, are found in the wall of OLs. Freeze fracture of OL membrane has revealed the presence of gap junctions connecting two OLs or OL and astrocyte. This coupling, via gap junctions, between perineuronal OLs and other cells is currently under investigation [162].

In 1979 Ludwin studied the remyelination process in mice. Perineuronal OLs were seen clearly remyelinating. Ludwin assumed that these cells may participate in normal myelination [173]. After a few years, he investigated the possible functions of perineuronal satellite OLs in the mouse; he used anti MBP and MAG and analysed OLs histochemically during the remyelination process. He concluded that these cells are functionally similar to other OLs in many respects. Ludwin stated that satellite OLs proliferate and produce myelin in response to injury or any demyelinating event [173].

It is believed that most of the functions that were attributed to OLs are thought to be functions of myelin rather than the cell itself [160, 240]. These include maintenance of axons, regulation of the axon calibre, and inhibition of axonal growth and regeneration [160].

4.2 Aims and hypothesis

OLs' numbers and function have proven to be essential in maintaining myelin and remyelination. Previous work revealed that cortical GM has more extensive

remyelination than the WM, which has been attributed to less inflammation [215]. Another animal study assumed that perineuronal OLs in the GM have an essential role in remyelination after injury [172]. Moreover, the significant role of perineuronal OLs in protecting neurons from undergoing apoptosis was also illustrated in a study by Taniike and colleagues in 2002 [240]. There is no study that investigated specifically the fate of OLs in the GM of MS.

Our aim is to study the pathology of OLs in the GM of spinal cords by comparing MS tissue and controls, and further comparing the two progressive subtypes of MS; PPMS and SPMS. Results obtained from counting OLs in the GM will be compared with results obtained from the WM in the following chapter. Axonal loss in NAWM has been confirmed previously [242] and found to correlate with the progressive form of the disease and with disability [127, 243], but the possible associated OLs pathology in normally appearing areas, especially the GM, has not yet been assessed.

4.3 Material and methods

Human spinal cord blocks were obtained for this study from the MS Society tissue bank in London (PPMS = 13, SPMS = 14, Controls = 5), Oxford Radcliffe NHS Trust (6 controls), and archival material from the Neuropathology Department of Nottingham University Hospital NHS Trust (7 controls) (Table 4.1). Details of the material from the MS Society were described as part of material in Chapter 2. Two controls from the MS Society tissue bank and 2 controls from Oxford were excluded because of inadequate staining (subjects ID numbers 11, 32, 3269, and 458).

For PPMS (4 males and 9 females) the age was between 45 and 92 years (mean = 66.23 years ± SD = 14.16 years) and the disease duration between 5 and 54 years (mean = 26.69 ± 14.57 years). For SPMS (5 males and 9 females) the age was

between 39 and 78 years (mean = 57.23 ± 13.59 years) and disease duration was between 9 and 50 years (mean = 29.62 ± 13.49 years). For control (5 males and 9 females) the age ranged between 33 and 93 years (mean = 64.15 ± 15.64 years).

Table 4.1: Neuropathology department of Nottingham tissue

Subject ID number	Gender	Age	Number of sections	Level of each sections
N8298	male	33	1	Cervical
NP4	female	60	2	Cervical, thoracic
NP47	female	72	3	Cervical, thoracic , lumbar
NP82 I	male	77	2	Cervical, thoracic
NP89 II	male	73	1	Thoracic
NP94	female	75	2	Cervical, lumbar
NP96	female	64	1	Thoracic

4.3.1 Initial preparation

Initial preparation of tissue from London and Oxford has been mentioned in Chapter 2 (Sections 2.5.1 and 2.5.3). To increase the number of controls, archival material from the Neuropathology Department in Nottingham University was added to our sample. Tissue from Nottingham was fixed in 10% formalin and embedded in paraffin. The formalin-fixed and paraffin-embedded blocks were cut at 5 μm thickness. Sections were processed for immunohistochemistry and stained against CA II. Immunohistochemistry protocol for CA II used the protocol mentioned in Section 3.7.2 and appendix B. Immunohistochemistry for tissue from Nottingham was carried out by another technician using a manual technique as opposed to the automatic immunostainer used for the tissue from London and Oxford.

Since we have two blocks from a number of subjects, the total number of blocks outnumbers the subjects. In total, 63 blocks of tissue have been analysed. From each block, one section was cut at 8 μm and the following 10 sections were cut at 5 μm thickness. The 8 μm sections were stained with H&E and the 5 μm sections were used for immunohistochemistry. The H&E stained sections were examined by an expert Neuropathologist Prof Jim Lowe and by an expert Consultant neurologist Dr

Evangelou Nikos. The H&E-stained slides were also compared with the CA II-stained slides. On the majority of slides, there was more than one section that can be examined. This gave us the opportunity to increase the number of the examined demyelinated lesions and to compare demyelinated and normally appearing spinal cord sections from the same subject.

Similar to the previous scanning process (Sections 2.5.3 and 3.3.3); the CA II-stained slides were numbered and converted into digital slides that can be viewed at the computer screen. Slides were viewed and edited on the computer by using the NDP.view software.

4.3.2 Sampling of the ventral horn and the dorsal horn

Using the digital pen, borders of the GM were delineated in all CA II-stained sections. The CSA of the cord and the GM were measured. From each VH, 5 fields were selected based on the protocol described in Figure 4.1. Fields were exported as an image of the computer screen when the magnification is set to 40X and the screen resolution is 1024 x 768 pixels. With this magnification and this resolution, the surface area of the exported field was 0.047 mm^2. However, when the VH area was small, especially at the upper cervical or thoracic levels, smaller fields were selected by exporting the image of the screen at magnification of 63X. The surface area of the field with 63X magnifications and with the same resolution was 0.0164 mm^2. The 63X magnification is set automatically by the software and cannot be manually adjusted. The majority of fields were exported at 40X magnification (65% of all fields).

The DH sampling protocol allowed for the field of interest to sample nucleus proprius (Figure 4.2). This means that the DH was represented by the Laminae IV and V. This is because lamina I is very thin and does not fill the field of interest and it is

separated from the nucleus proprius (lamiae IV and V) by the naturally unmyelinated substantia gelatinosa (laminae II and III).

4.3.3 Oligodendrocyte counting

Each exported field of interest was given a number so that the observer is blinded to myelin status and the disease type. Fields of interest were numbered based on the subject ID number, section number, side of tract (left or right) and its location among the 10 sections. Therefore, each field location can be traced and located on the original slide. All fields were viewed by imageJ software. The field was opened using the software and manual counting was carried out. Using the computer mouse cursor, all OLs were selected. The software automatically counted the selected points. Reliability of our identification criteria and reproducibility of our counting process was tested (coefficient of variation 16%).

4.3.4 Identification of the myelin status of fields

To determine the myelin status of each field, the MBP-stained sections were used. However, the MBP and CA II sections are not adjacent. The two non adjacent sections were selected randomly from 10 consecutive 5 μm thick sections. Thus the maximum distance between any two sections can reach up to the thickness of 8 sections (40 μm). The effect of the possible maximum 40 μm distance between the MBP and the CA II sections on our measurements was tested by measuring the surface area of the spinal cord and the GM in 10 subjects and in the three stains (CA II, MBP, and H&E).

The CSA and the GM surface area were calculated in the three stains; CA II, MBP, and H&E (Table 4.2). The correlation of GM surface area between CA II and MBP was 0.992 (p value < 0.001) and with H&E was 0.995 (p value < 0.001). The WM

surface area calculated from the CA II and the MBP correlated with each other significantly (Pearson correlation r = 0.990, p value < 0.001). The GM surface areas calculated from the CA II sections correlate also with MBP (Pearson correlation r = 0.996, p value < 0.001).

4.3.1 Classification process of CA II-stained fields according to myelin status

Using NDP.view software, the CA II-stained image was opened and then the corresponding MBP-stained image was opened on the same screen. By synchronising the two images using the NDP software, magnification and position of the two opened images were set equal, ie any change in the magnification or position of the first image was met by the same degree of change in the magnification and position of the second image.

Table 4.2: The GM and the WM surface areas in the three stains

The subject ID	The WM surface area (CA II)	The WM surface area (H&E)	The WM surface area (MBP)	The GM surface area (CA II)	The GM surface area H&E	The GM surface area MBP
E49	30.2	29.4	28.0	4.11	4.08	3.74
E55	51.0	51.0	50.8	2.58	2.31	2.48
E72	24.9	25.0	25.0	3.38	2.41	3.29
E104	42.6	42.2	43.1	3.36	3.36	3.50
E109	35.5	35.3	35.8	4.18	4.29	4.16
E122	42.6	44.5	44.7	9.21	9.08	9.73
E135	32.2	32.8	33.1	12.23	12.70	12.29
E164	30.4	30.1	30.1	3.59	3.40	3.28
E197	23.4	24.0	20.6	14.09	13.43	12.52
E49	30.2	29.4	28.0	4.11	4.08	3.74

The only feature that was not included in synchronisation is the orientation, which was adjusted before synchronising the images. This was because each slide had been scanned in a different orientation by the NDP.view scanning machine. Orientation of each slide was standardised by drawing a reference line. The reference line was drawn from the central canal to the dorsal border of the PCP. To adjust and standardise the orientation, the line was adjusted to one of the vertical

borders of a flat computer screen. Therefore, each time the examiner opens the two images, he can retain the image alignment by adjusting the line to the vertical border of the computer screen. Once the orientation of the two opened images (CA II and the MBP of the same clock) was the same, images were then synchronised. Synchronisation of the two images allows the examiner to compare the same region in two different stains (Figure 4.3).

Because the previously exported CA II-stained fields were still outlined on the CA II-stained sections, the same regions could be located on the MBP when the two images were synchronised. Consequently, the myelination status of the fields was then identified (Figure 4.3). Fields were classified into normally appearing GM (NAGM), partially myelinated GM (PMGM), and demyelinated GM (DMGM). DMGM included all the completely demyelinated areas. Any abnormally appearing areas that were not completely demyelinated were considered PMGM. Therefore, PMGM included the lesion border, areas of partial remyelination or areas of partial demyelination.

4.3.2 Statistics and Statistical software

Results were exported to SPSS software that has the subject number, the field number, OLs number, CSA of each field, and the myelin status of each field. The spreadsheet also has other details about the subject's age, gender, duration of the disease, and type of disease.

Reproducibility and reliability were evaluated by coefficient of variation and Spearman correlation. T test and Mann-Whitney U test were used to compare means as appropriate. Multiple linear regression was used to analyse effects of independent factors on OLs, such as age, gender, type of disease, cord level, duration of disease

and post-mortem delay. With the help of Dr Owen, a lecturer at Nottingham University, comparison of the means and regression analysis were double checked using STATA statistical software.

4.4 Results

About 864 fields were examined (Table 4.3). Some of the general features of CA II stained sections were mentioned in the Chapter 3, while MBP stain features were mentioned in Chapter 2. GM OLs appeared variable in shape and size. In controls, they appeared homogeneously distributed throughout the GM. The electron microscopic features of OLs having heterogeneous chromatin were noted frequently [169]. The discrimination between light, intermediate and dark OLs was not always possible. However, in agreement with the electron microscopic findings, it was noticed that darker OLs tend to be smaller. Presence of large light OLs, which represent early mature cells, was often observed (Figure 4.4 A). Perineuronal OLs were occasionally seen in proximity with the perikaryon of neuron, and they tended to be darker and smaller (Figure 4.4 B). In the lesions' centres the presence of OLs was not associated with myelin.

Table 4.3: Distribution of the analysed fields from the VH and DH

Type of the disease	Myelin status of the field	Number of fields
Normal control	Normal field from normal control	250
PPMS	Demyelinated	51
	Partially myelinated	51
	Normally appearing fields from MS	219
SPMS	Demyelinated	58
	Partially myelinated	29
	Normally appearing fields from MS	191

4.4.1 The spinal cord grey matter of MS shows higher density of oligodendrocytes than normal controls.

The mean density of OLs in the GM of MS, regardless of disease type and myelin status, was 139 ± 159/mm^2 and in controls was 104 ± 96/mm^2 ($p < 0.001$) (Figure 4.5). We further examined the mean density in each of the main cord segments (the three main segments; cervical, thoracic, and lumbar). In both controls and MS subjects, there was no significant difference in OLs' density between any of the three segments. In addition, regression analysis did not reveal significant effect of cord level on GM OLs in either MS or controls.

4.4.2 The spinal cord grey matter of SPMS has greater density of oligodendrocytes compared to PPMS

By considering the disease type, the mean density of OLs in the GM of PPMS was 122 ± 136/mm^2 and in SPMS was 158 ± 180/mm^2 ($p = 0.007$). When each subtype is compared with controls, only SPMS showed significant difference (comparison between controls and SPMS, the p was < 0.001 and with PPMS, the $p = 0.064$) (Figure 4.6).

The increase in OLs in SPMS GM compared to PPMS GM was observed only in the VH. The mean OLs density in the VH of SPMS was 164 ± 182 and in PPMS was 123/mm^2 ± 132 ($p = 0.006$). In the DH, the mean OLs density was 135 ± 174/ mm^2 in SPMS and 117 ± 151/mm^2 in PPMS ($p = 0.548$).

4.4.3 Density of oligodendrocytes in the NAGM

When we further sub-classified our analysis according to myelin status, we found out that the increase in OLs was due to increase of numbers in the NAGM. The mean density of OLs in the NAGM of MS was 173/mm^2 ± 7.99 (compared to controls the p

< 0.001). OLs' density in the NAGM was found to be 149/ ± 133/mm^2 in PPMS and 201 ± 186/mm^2 in SPMS (p = 0.002). This increase in OLs' density in the NAGM of SPMS was found to be due to increase in OLs in NAGM of the VH and not the DH. The OLs' density in the NAGM of the VH in SPMS was 207± 187/mm^2 and in PPMS 149 ± 128/mm^2 (p = 0.001). In the NAGM of the DH, OLs' density was 178 ± 185/mm^2 in SPMS and 151± 152/mm^2 in PPMS (p = 0.463).

4.4.4 Density of oligodendrocytes in PMGM

The mean density of OLs in PMGM was 130 ± 165/mm^2, which was significantly less than OLs' density in the NAGM (p = 0.037) and not different from healthy GM of normal controls (p = 0.183). In the PMGM, PPMS showed a density of 124 ± 154/mm^2 and SPMS showed a density of 142 ± 187/mm^2 (P = 0.65). Comparison between the DH and the VH in the PMGM was not possible because there were not enough PMGM fields exported from the DH. Most of the lesions that affected the dorsal aspect of the cord resulted in complete demyelination of the DH.

4.4.5 Density of oligodendrocytes in DMGM

OLs' density in the DMGM was found to be tremendously less than normal healthy controls (83% reduction) and less than PMGM (86% reduction) and NAGM (90% reduction). The mean density of OLs in the DMGM of MS was 17 ± 39/mm^2. In SPMS the density was 28 ± 49/mm^2, which was significantly more than the DMGM in PPMS that showed OLs' density of 4.8 ± 15/mm^2 (p = 0.001). In the whole MS sample and the two forms of the disease, the demyelinated areas showed significantly reduced density of OLs compared to healthy GM from normal controls, NAGM and PMGM.

4.4.6 Effects of age and disease duration on GM oligodendrocytes numbers

In normal controls, GM OLs' number decreases significantly with age ($p < 0.001$, correlation coefficient = -0.413 controlling for gender and cord level). However, effect of age on OLs' density in MS was found to be insignificant controlling for gender, myelin status and cord level (Correlation coefficient = -0.013, $p = 0.748$).

In MS, correlation of OLs' numbers in the GM correlated better with duration of disease than age. Duration of the disease seems to have weak negative correlation with number of GM OLs in the global sample. However, effect of duration of the disease on OLs was only significant in the demyelinated areas in which the correlation coefficient was calculated to be -0.221 and the significance was 0.026 controlling for age, gender, and cord level (Table 4.4).

Table 4.4 correlation of OLs density with disease duration in the three differently myelinated region

Myelin status of the field	Control Variables		Disease duration in years
Demyelinated	Gender & Level of the cord & Age	Correlation	-0.221
		Significance (2-tailed)	0.026
Partially myelinated	Gender & Level of the cord & Age	Correlation	-0.121
		Significance (2-tailed)	0.296
Normally appearing from MS	Gender & Level of the cord & Age	Correlation	-0.021
		Significance (2-tailed)	0.680

4.4.7 Total number of oligodendrocytes in the spinal cord GM

To validate our results further and to overcome the possible effects of atrophy in MS on OLs' density, which may give misleadingly higher density of OLs per 1 unit surface area, the total number of OLs was calculated in the GM. This has been calculated by multiplying the mean density of GM OLs in each subject by the GM surface area of that subject. Only the VHs that do not have demyelination were included in assessment of OLs total number. Due to significant variability in the GM

volumes among various cord levels, results were categorised into 5 groups according to the GM volumes in each segment (Sections 2.5.5 and 2.7.2).

The total number of OLs in the upper cervical GM of controls was 449 compared to 746 in MS (Mann-Whitney U test = 4, p 0.643). In the lower cervical GM, which has great surface area, the total number was 942 in controls and 453 in MS (Mann-Whitney U test = 5, p value = 0.194). In the thoracic area, the total number was 363 in controls and 648 in MS (Mann-Whitney U test = 13, p = 0.295). In the upper lumbar GM, the total number was 338 in controls and 967 in MS (Mann-Whitney U test = 2, p = 0.857). The total number in the lower lumbar level was 1639 and 2260 in controls and MS respectively (Mann-Whitney U test = 13, p = 0.295).

4.4.8 Comparison between the dorsal horn and the ventral horn

We demonstrated previously that the difference between PPMS and SPMS was only significant in the VH. However, comparison was made between OLs' density in the ventral GM and the dorsal GM in both controls and MS. In healthy controls, there was greater density of OLs in the VH (109/mm^2) compared to the DH (54/mm^2) with a significance of < 0.001. The variation was less prominent in the global sample of MS where the VH and the DH demonstrated mean densities of 142/mm^2 and 126/mm^2 respectively (p = .319). Table 4.5 shows the variation according to the myelin status.

4.4.9 Effects of independent factors

A linear regression model was applied to study the effect of the independent factors (disease duration, gender, level of the cord, myelin status, type of the disease, region of the GM (VH vs DH) and post-mortem delay) on the dependent factor OLs' density. There was significant effect of disease type and myelin status on OLs' density and p value were 0.029 and < 0.001 respectively.

Table 4.5: Comparison between the VH and the DH in oligodendrocytes density

Type of sample		GM	OLs density / mm^2	P value *
controls		VH	109/mm^2	<.001
		DH	54/mm^2	
MS	NAGM	VH	175/mm^2	.603
		DH	165/mm^2	
	PMGM	VH	128/mm^2	**
		DH	171/mm^2	
	DMGM	VH	21/mm^2	.063
		DH	5/mm^2	

* paired t test

** Only 3 partially myelinated fields from the DH were found in the sample and thus comparison was not possible. As we have already mentioned, most of the lesions were posterolateral and affected the whole DH.

4.4.10 Summary of results

So far, we have calculated that the mean density of OLs in the GM of MS is more than controls in the whole sample and in SPMS. When the analysis is extended to consider the myelin status, the NAGM of MS in the whole sample and in both subtypes demonstrated greater density of OLs than healthy GM of controls. Increase in OLs was found in the NAGM or the lesion border, while in the completely demyelinated areas number of OLs is significantly reduced. There are more OLs in the NAGM, PMGM and DMGM of SPMS cords compared to PPMS. This difference was greatest in the NAGM of the VH. In normal controls, GM OLs' number reduces with age, while in MS the number of OLs reduces slowly with disease duration in the demyelinated areas.

4.5 Discussion

Exporting fields of interest from the spinal cord GM using unbiased sampling protocol is, to our knowledge, the first quantification study of OLs in the GM of MS. OLs are responsible for an important element of CNS repair, which is the remyelination. The CNS remyelination occurs in the WM and the GM. Effective remyelination depends on the presence of enough functioning OLs [217, 244, 245]. We have calculated OLs' density in four areas; healthy controls GM, NAGM, PMGM, and chronic DMGM. All

lesions in our sample were chronic demyelinated lesions. Inflammation stages of lesions in this sample have been studied previously using CD3 and CD163 and the majority of lesions were found to be chronic inactive with very low inflammation score [124]. This is an expected result, as new lesions of the spinal cord in progressive MS are quite rare [138].

Interestingly, we found high numbers of CA II positive cells in the GM of the entire MS sample compared to controls. We found that the NAGM is responsible for this increase. When the two subtypes of the disease were compared, the density of OLs in the GM of SPMS was greater than that of PPMS. The highest number of OLs was found in the spared regions of the GM of the two disease forms.

It is possible that GM lesions are associated with some activity in the form of inflammation and substantial release of inflammatory molecules or chemotactic signals. This may affect the OLs around the lesion. OLs may undergo proliferation in response to a signal from the lesion. OLs' proliferation in response to injury has been reported on a number of previous occasions. Animal studies have reported GM OLs' proliferation after nervous tissue injury [246]. Experimental spinal cord injuries in rats have shown that new OLs are found around the lesion border as early as 7 days post injury, The numbers of accumulating OLs around a spinal cord lesion was found to be greater than in normal controls [226]. Surprisingly, OLs' proliferation resulted in high numbers of OLs in the GM that outnumbers GM OLs in controls. Proliferation was also more prominent in the GM than the WM [226]. Our results suggest that this finding may also occur in the human nervous system.

The observation that spared areas around lesions have the highest numbers of OLs led us to execute a quick comparison between density of OLs in the spared areas

near GM lesions and spared areas far from lesions. We found that NAGM has significantly greater density of OLs in the cervical segment (199/mm^2), which is nearer to lesions compared to lumbar segment, where lesions are rare (158/mm^2) (p value = 0.04). This may support further that OLs proliferate in response to the local effects of demyelinating lesions.

The role of the presumed OLs' proliferation in the GM is not certain. The increased numbers of OLs in the NAGM may act as a reservoir for OLs. OLs' progenitors possibly proliferate and migrate to injured areas. These OLs accumulate around the border, in an attempt to migrate inside the lesion and repair it or to prevent the lesion from spreading further. This may explain the finding that SPMS has more OLs in both the NAGM and the DMGM.

An important issue is that comparison with animal studies may not be ideal. Animal studies mostly test acute response to injuries of the spinal cords compared to the chronic lesions of MS, which have been examined in our study. Nevertheless, a human study in MS showed that proliferation of cells continues in chronic lesions of MS [247].

Another important concern is the possible effect of atrophy of the spinal cord in MS on interpretation of results. Atrophy of tissue in MS, including the GM, may result in overestimation of OLs' density. In an attempt to validate our results, the total number of OLs within the cord was calculated, which showed that there is no significant difference between MS and controls. It should be noted that we included only normally appearing sections that do not have demyelination. This resulted in exclusion of sections with demyelination, and consequently exclusion of the areas around the lesions, which are the areas with the highest number of OLs. On the other

hand, including sections with demyelination in the analysis of total numbers of OLs may not be reliable. This is because significant areas of these sections are occupied with demyelination, which have very few OLs.

There may be another possible source of error coming from controls. The control sample was obtained from three sources and may give variable results. Initially, we had two sources of tissue; London and Oxford, which have been processed in the same lab and by the same technician. Initial results revealed that there was an increase in OLs' density in the GM of MS compared to controls. To increase the reliability of our results, we added several controls from Nottingham. For technical reasons, these were processed manually by another technician and using manual technique rather than automatic technique (immunostainer). The manual technique has used the same protocol that had been done by the immunostainer. The obtained results did not vary considerably and controls GM continue to demonstrate less numbers of OLs compared to MS GM. However, both Oxford tissue and Nottingham tissue demonstrated greater density of OLs in the GM compared to the tissue from London.

4.5.1 Variation between PPMS and SPMS

MS tissue was obtained from one source and processed by the same technician. Therefore, comparison between PPMS and SPMS is not affected by source of tissue variation. SPMS spinal cords seem to have higher density of OLs in the NAGM in contrast to PPMS. If we move inside the lesion, OLs number reduces tremendously. But still OLs density in SPMS lesions was greater than PPMS (in SPMS was 27 OLs/mm^2 and in PPMS was 5 OLs/mm^2). This may indicate that OLs in the NAGM migrate inside the lesion or the process of production of OLs is more efficient in SPMS compared to PPMS. Previous studies have reported more OLs' precursors in

SPMS compared to PPMS. The density was 34/mm^2 in lesions from SPMS and 9/mm^2 in lesions from PPMS [239].

Variation in OLs pathology between MS subtypes may be part of the variation in the whole disease process. There are differences between the two subtypes in degrees of inflammation, atrophy and in demyelination. The progressive phase of SPMS patients can be shorter than PPMS, which may explain the findings that SPMS maintains more inflammation [103], more inflammatory cell recruitment, more demyelination and even more remyelination [179]. In SPMS, there was more perivascular cuffing and cellularity in the parenchyma of SPMS [103]. There were also more T cells and activated microglia inside and outside spinal cord lesions in SPMS [124]. Part of OLs' response to inflammation may include proliferation or migration to the site of inflammation and, as an element of this inflammation, OLs' recruitment may be higher in SPMS. This may indicate that SPMS by having more inflammation attracts more OLs to the site of inflammation. In fact, an experimental animal study reported that inflammation is a potent stimulator of OLs proliferation and remyelination [248]. Specifically in MS, Lucchinetti et al reported that SPMS ability to recruit inflammatory cells and OLs to the site of lesion was greater than PPMS [179]. This is supported by a previous report that SPMS patients have greater degree of remyelination than PPMS [249].

In Chapter 2 we demonstrated that SPMS has more demyelination in the spinal cord, but PPMS has greater tendency to affect the GM and may therefore produce greater damage to OLs, which may result in reduced numbers of OLs in the GM of PPMS. Moreover, variation in atrophy between the two disease subtypes may affect interpretation of our results. We found that SPMS produces more atrophy of the cord, especially in the upper segments, which may give misleadingly higher density of OLs

and thus explain the higher density of OLs in the SPMS GM. This possibility is further suggested by the fact that there was no significant difference between SPMS and PPMS in the total number of OLs in the GM. Again, we have to emphasise that sections with demyelination, where OLs accumulate around lesions, have been excluded from the study of total number of lesions. Therefore, the main contributory element to the increase in OLs number is not included.

Another explanation is that SPMS subjects, due to genetic, environmental or other unknown factors, may have greater resistance to OLs loss. As a result, these cells can be mature OLs that survived demyelination. It was observed that in some MS lesions OLs were relatively preserved. These OLs were found to be positive for Bcl-2. This protein protects against OLs apoptosis. Expression was found in demyelinated areas, but highest in remyelinated areas. A subset of patients with RRMS demonstrated high numbers of Bcl-2 positive OLs [250]. It is possible that RRMS subjects maintain expression of this protective protein in the secondary progressive phase of the disease. Such assumption was further supported by Bonetti et al in 1997, reporting that similar expression of Bcl-2 was noticed in chronic active and in silent MS lesions [251]. Therefore, future studies may compare expression of Bcl-2 between SPMS and PPMS, and between the GM and the WM.

4.5.2 Potential sources of oligodendrocytes

So far, we have suggested two possible sources of OLs; new OLs from proliferation of the progenitor cells or mature OLs that survived demyelination. However, it is strongly believed that remyelinating OLs in the human CNS are new OLs that are produced by differentiation and proliferation of OL progenitors [194, 252-254]. There are two pieces of evidence to support this hypothesis; adult OLs that survive the demyelinating event are mature and therefore postmitotic (mitotically inactive), and

new OLs were found mainly in areas populated with NG2 cells [226]. Animal studies have demonstrated that mature OLs that survived demyelination do not play a role in remyelination and that new OLs are required for remyelination [255]. In fact, the numbers of mature OLs that survive demyelination in MS (sometimes called demyelinated OLs) reduces significantly with disease progression [238].

Moreover, Solanky et al (2001) studied 3 groups of patients; patients with MS, other neurological diseases, and normal controls. The study quantified proliferating cells by using proliferation marker (Ki-67) that stains cells in various phases of cell cycle. There was more proliferation of cells in MS. Most of the proliferating cells were found to be OLs and were traced in acute, chronically active, and chronic lesions. The study demonstrated that OLs' proliferation continues even in chronic lesions [247]. Sources of new OLs may include, NG2 positive cells, PDGF-α positive cells, nestin positive cells, and perineuronal OLs (Table 5.4).

Adults NG2 positive cells

Adult NG2+ cells are different from embryonic NG2+ cells, in that embryonic NG2+ precursors differentiate early during gliogenesis and are restricted to a certain area around the neural tube. The exact function of adult NG2+ cells is not clear, but it is believed that these cells are part of OLs' lineage and they can differentiate into OLs in pathological cases such as MS [194, 226].

Adult NG2 positive cells are a distinct population of cells that are distributed evenly in the adult human CNS in the GM and the WM. NG2 positive cells can be found in developing brain, adult brain, and in chronic lesions of MS [237, 244, 245]. These cells express PDGF-α, and O4, but not MBP or CNPase. These NG2+ cells are capable of producing OLs in response to injury, which is a rapid and efficient process

for remyelination in animals [194]. It was found that these cells proliferate early in response to injury (within the first week), and are capable of producing new OLs around the lesion border as early as 3 days post injury. Newly formed OLs were observed in areas rich with NG2+ cells [226]. Animal studies revealed that NG2 positive cells are the remyelinating cells and their exogenous transplant in another lesion promotes remyelination [245].

After injury, NG2+ cells of the spared areas change their morphology from stellate shape to a more regular shape. This regular shape is believed to be the active proliferating form of the cell [256]. The shape of premyelinating OLs that was found in chronic lesions of MS in previous studies and the present study greatly resembles the active form of NG2 positive cells [236]. They were oval cells and were not associated with myelin. NG2 cell distribution correlates positively with the distribution of premyelinating OLs indicating that NG2 cells give rise to premyelinating OLs [236]. Another study compared OLs pathology in EAE and MS [252]. It demonstrated that NG2 cells proliferate and become active in response to injury and a transition state between NG2 and mature OLs was found in MS, where double stained cells with NG2 and CNPase were found [252].

PDGF-α positive cells

These cells are earlier than NG2 positive cells in cell lineage of OLs. Although double labelling with NG2 is common, these PDGF-α positive cells are different from adult NG2 positive cells by being bipolar or sometimes with no processes. An important feature of these cells is that they are migratory. During normal development, the bipolar PDGF-α positive cells differentiate into embryonic NG2 positive cells and then to premyelinating OLs or to adult NG2 positive cells. Adult NG2 cells continue as the distinct population of cells mentioned in the previous section, while premyelinating

OLs are not present normally in adult brains. Premyelinating OLs are temporary cells. During early development, the multiprocessed premyelinating OLs either develop into mature myelinating OLs or undergo apoptosis within 3 days, if they do not myelinate axons [194, 223, 227]. Multiple processed premyelinating OLs have been observed in chronic lesions of MS [236].

During development of the spinal cord, OLs' proliferation begins in the ventral part of the central canal. Two foci of PDGFR-α positive OLs are generated on both ventral sides of the neural tube and then OLs' proliferation is started [257]. The dorsal spinal cord was to found to be inhibitory for proliferation of OLs in the early stages of development [224]. Animal studies revealed that proliferation of OLs was more apparent in the VH [258]. In our sample, the observation of accumulation of OLs at the border of GM lesion was only noted in the VH. In addition, the variation between SPMS and PPMS was only noticed in the VH.

PDGFR-α positive cells were identified in 1995 in a cultured human temporal cortex [259]. After that, and in 1998, Neil Scolding and his colleagues examined normal controls, acute, and chronic MS lesions for the presence of these cells. They demonstrated for the first time the presence of small numbers of PDGF-α positive cells in the brains of normal controls, in acute and in chronic lesions of MS [245]. This may represent another potential source of OLs in MS. From the data in our hand, we calculated that in controls there are higher numbers of OLs in the VHs compared to the DHs. Comparison between the ventral and the dorsal spinal cord is important, because of the observation that most of spinal cord lesions are located posterolaterally in the spinal cord.

Nestin positive cells

These cells are the neuronal stem cells and they are earlier than NG2 and PDGF-α positive cells. They are rounded-to-oval non-branching cells that can give rise to neurons and glia. They are positive for nestin and localised to the sub-ventricular zone during development. Neuronal stem cells are not different morphologically from PDGFR-α positive cells, as some authors describe PDGFR- α cells as non-processed cells. The difference is that PDGFR-α cells are committed to become OLs. Presence of these cells in the adult human CNS has been recently reported and they were localised around the central canal of the spinal cord near to ependymal cells [260]. These cells may be an important potential source of new OLs, especially because they are characterised by being multipotent and migratory [160].

Perineuronal oligodendrocytes

Another possible source of new OLs in the human GM is the perineuronal OLs. It has been suggested that perineuronal OLs play a role in remyelination in animals. Perineuronal OLs proliferate, differentiate and remyelinate injured axons in animals. In the GM perineuronal OLs, which are not associated with myelination in normal circumstances, were seen clearly remyelinating axons [172, 173]. This may explain the presence of high numbers of OLs in the GM of MS spinal cords.

4.5.3 Further Comments on material and methods

There are limitations to the use of the human tissue in examining diseases such as MS. For example, duration of the disease varies at time of death and, therefore, age of lesions may vary. Although most of our sample subjects had a similar degree of disability before death [124], pathology of GM and thus OLs changes may be independent of disability. Another limitation may be related to a different post-mortem delay of tissue. This may affect the degree of protein and enzyme expression.

Myelin status was determined by the MBP-stained sections. Because the CA II-stained section and the MBP-stained section are not adjacent, a possible error may arise from identification of the myelin status of the field, such as misinterpretation of DMGM as NAGM or vice versa. The maximum possible distance between two sections is 40 μm. When we calculated the surface areas, there was strong correlation between the two images, which indicates that they are very close to each other. In addition, we have considered any abnormally appearing area that is not completely demyelinated as PMGM. As a consequence significant surface area around the border was included as partially myelinated areas. Having a wide border included as PPMS reduces the risk of misinterpretation of demyelinated areas as normally appearing areas. We also calculated that the mean size of spinal cord lesions in the cross sectional plane was 13 mm^2 and therefore the effect of 40 μm in the longitudinal plane of the spinal cord is not expected to be significant, especially because most spinal cord lesions run in the longitudinal plane of the spinal cord. In fact, for many sections, the lesion border can be identified on CA II stain at high power when synchronised with the corresponding MBP-stained section. The border is very difficult to identify on CA II-stained images alone without synchronising the image with the corresponding MBP-stained image. Consequently, we were confident that identification of myelin status using the previous method was accurate and did not affect interpretation of results.

4.6 Conclusion

There are high numbers of OLs in the GM of MS. The origin of these OLs is uncertain, but animal and human studies have suggested that these cells are new OLs. Four sources of new OLs are suggested; from adult NG2 positive cells, PDGF-α positive cells, Nestin positive cells, and from perineuronal OLs. These possible sources of OLs may proliferate to premyelinating OLs within the lesions or migrate

from the nearby areas. In the WM chronic lesions of MS, the present OLs are premyelinating immature cells that are positive for O4, NG2, PDGF-α and CA II, and negative for MBP, CNPase and PLP. Therefore, identifying level of maturity of CA II-positive cells in the future studies is essential.

There is little information about the degree of spinal cord GM demyelination and its effects on disability. We also have little information about the degree and extent of remyelination in the spinal cord GM, and whether remyelination of the GM plays a role in improving patient's symptoms and how this may affect disease progression. Our study showed that in GM lesions, CA II-positive cells in GM reduces slowly but significantly with chronological progression of the disease.

We also demonstrated that SPMS has more OLs in the NAGM and the DMGM. However, the number of cells within the lesion reduces with progression of the two disease forms. This has been further confirmed in this study where the duration of the disease significantly affects the number of OLs in the demyelinated areas. This may indicate that MS lesions in PPMS and SPMS lose the receptive environment for OLs proliferation, differentiation or migration [244]. Another explanation may be the physical limitation caused by the astrocytic scar or loss of signal from the degenerated axons due to long standing demyelination.

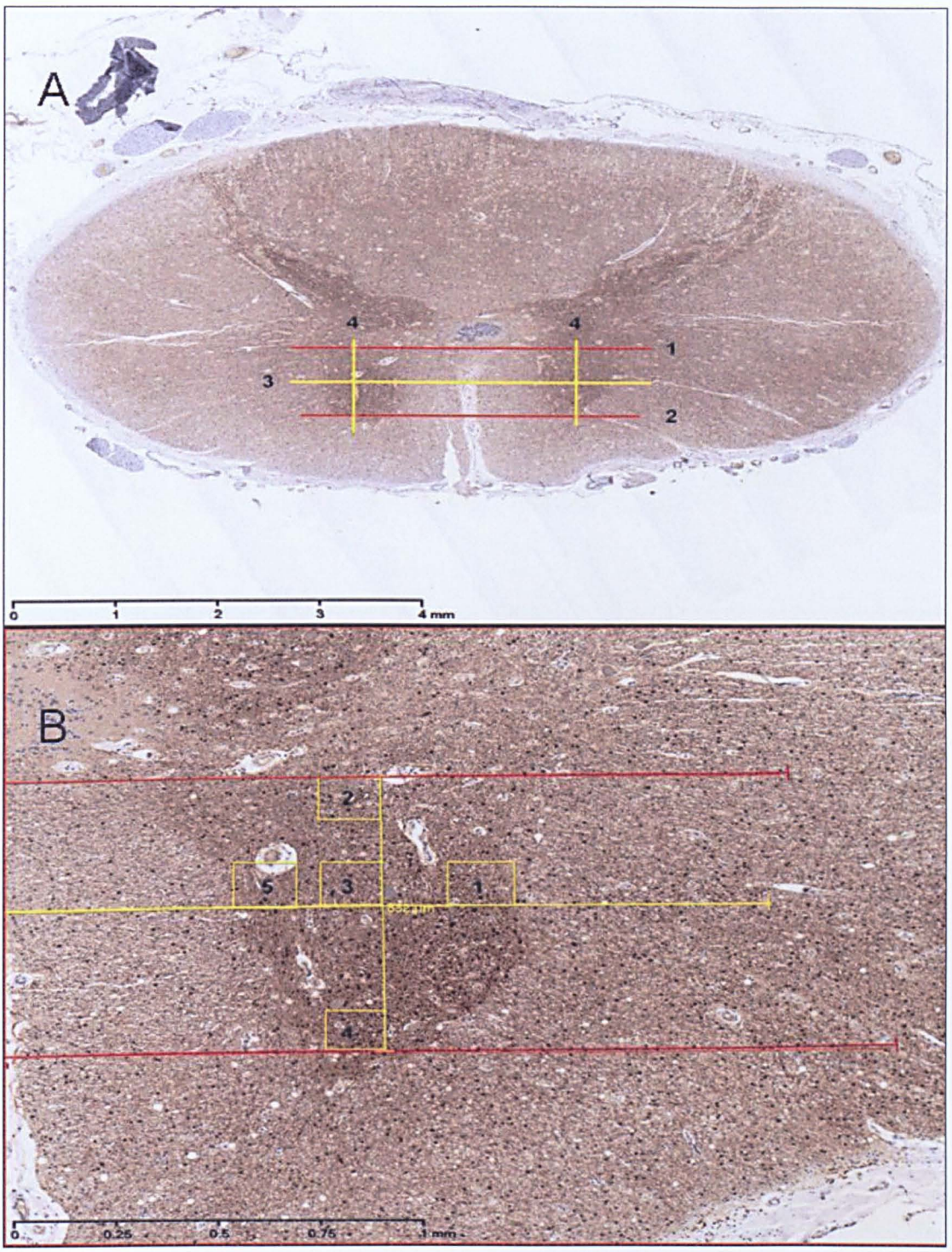


Figure 4.1: Sampling protocol of the VH. From each side, a horizontal line was drawn from the anterior border of the GM commissure (line 1). A second horizontal line was drawn between the tips of the VHs (line 2). A third horizontal line was drawn in the middle between line 2 and 1 (line 3). The intersections of the third line with the VHs boundaries were marked on both sides. The midpoint between the two intersections was identified. A vertical line was drawn through the midpoint on each side, which divided the VH into 4 quadrants (line 4) (A, scale bar 4 mm). Fields were selected from 5 regions of each VH at a magnification of 40X or 63X (B, scale bar 1 mm).

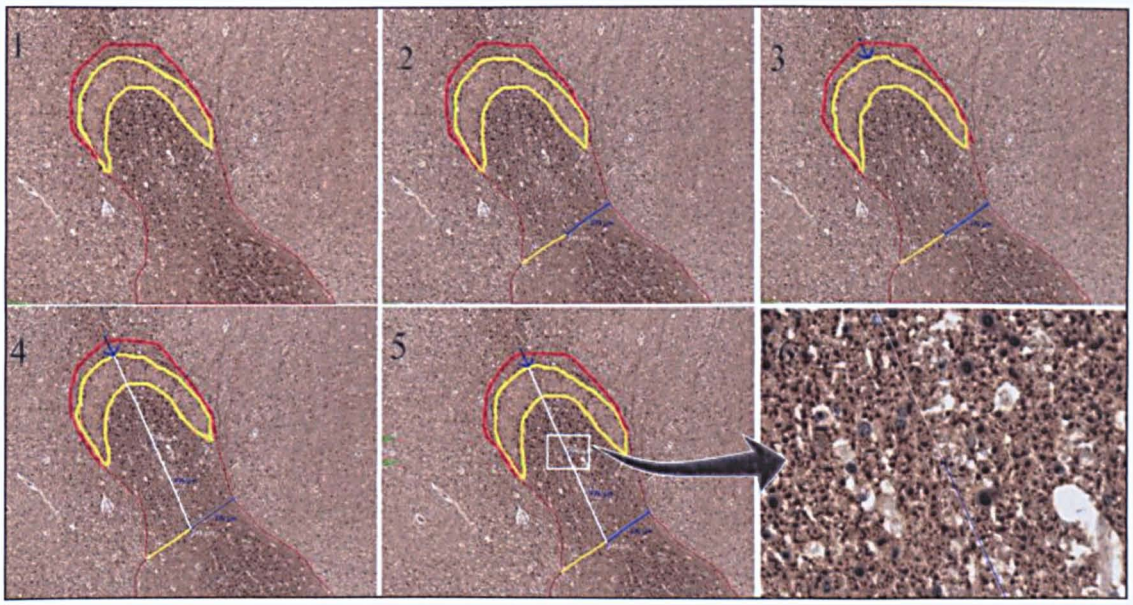


Figure 4.2: The sampling protocol of the DH. In 1, the DH is demarcated and a transverse line is drawn through the neck of the DH in 2. The midpoint of this line is identified in step 3. In 4, a second line is drawn from the midpoint of the first line to the tip of the outer border of the substantia gelatinosa. In 5, the midpoint of the second line is centralised in the middle of the screen. Finally, the image magnification is changed to 40X and then the whole field that appear on the screen is exported.

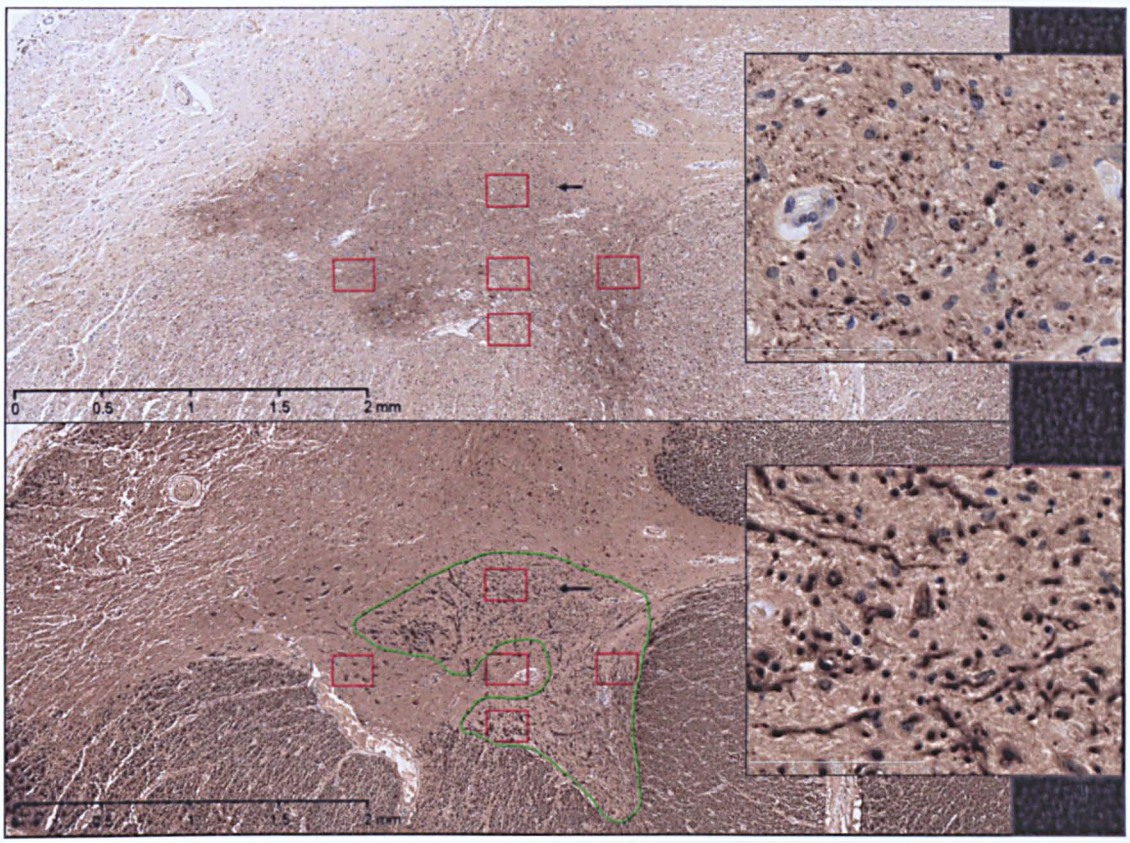


Figure 4.3: Two synchronised images as they appear on the computer screen. If the examiner magnifies the pointed field in the upper image, same region in the lower image is automatically magnified. Note the myelin status of the area (arrow) seen with MBP and how the same area appears with CAII above. It can be noticed that areas of demyelination and partial myelination are not identifiable on CA II sections. The GM boundary is more prominent in CA II-stained sections in the presence of severe demyelination compared to MBP-stained section (scale bar 2 mm)

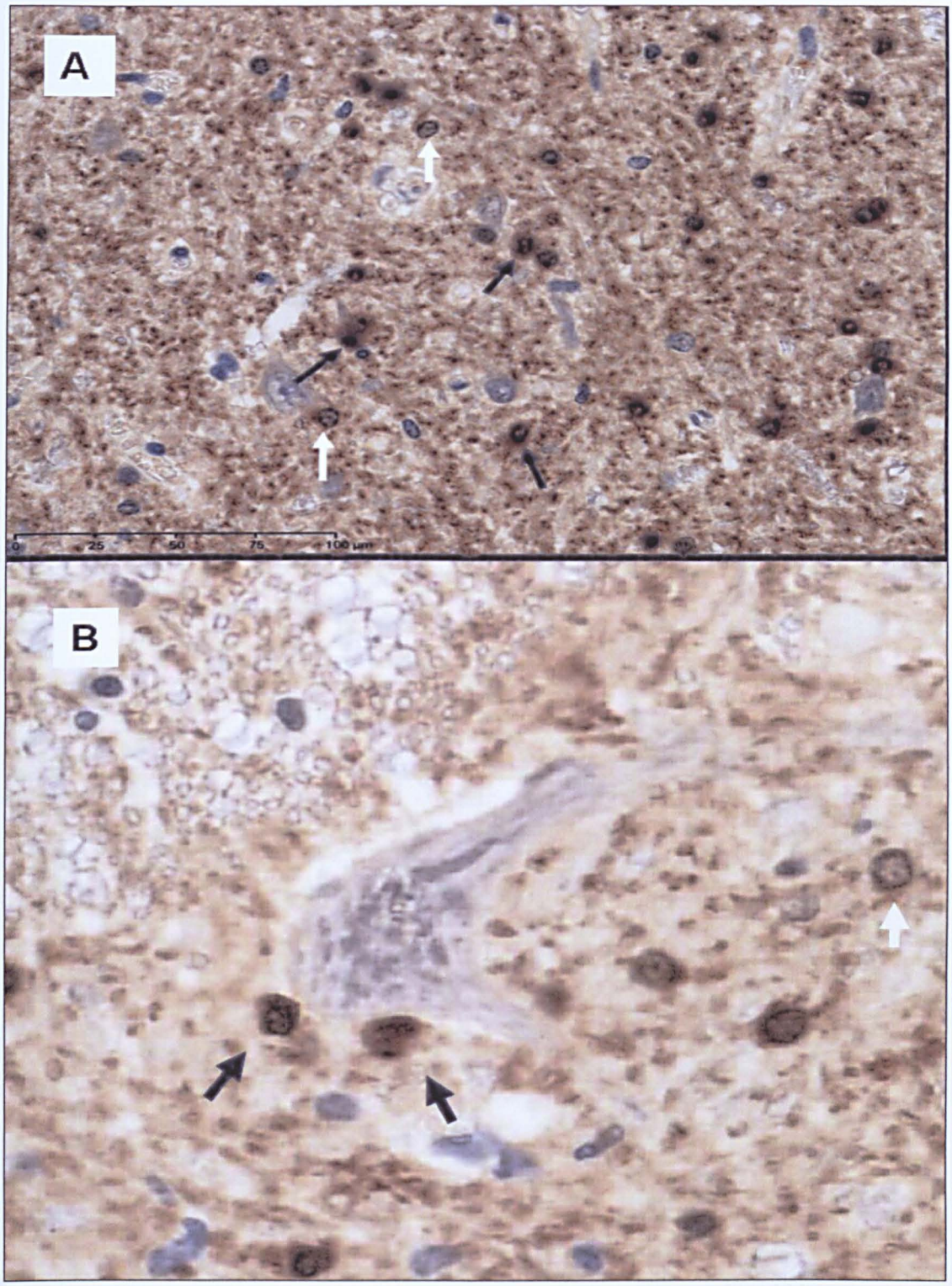


Figure 4.4: In A, OLs in the GM of human spinal cords stained with CA II marker. Field is 40X magnification. Black arrows point to three possibly dark OLs. White arrows point to light OLs, which mostly represent less mature OLs. The condensed chromatin at the rim of the nucleus is obvious and considered as one of the characteristic features of OLs. All other small particles are possibly due to myelin staining. The H&E counterstained cells (blue cells) are possibly other glial cells or small interneurons. (Scale bar 100 µm). In B, the black arrows point to two perineuronal OLs in close proximity to the cell body of a neuron in the GM. Perineuronal OLs staining was more intense than non-perineuronal OLs (100X magnification).

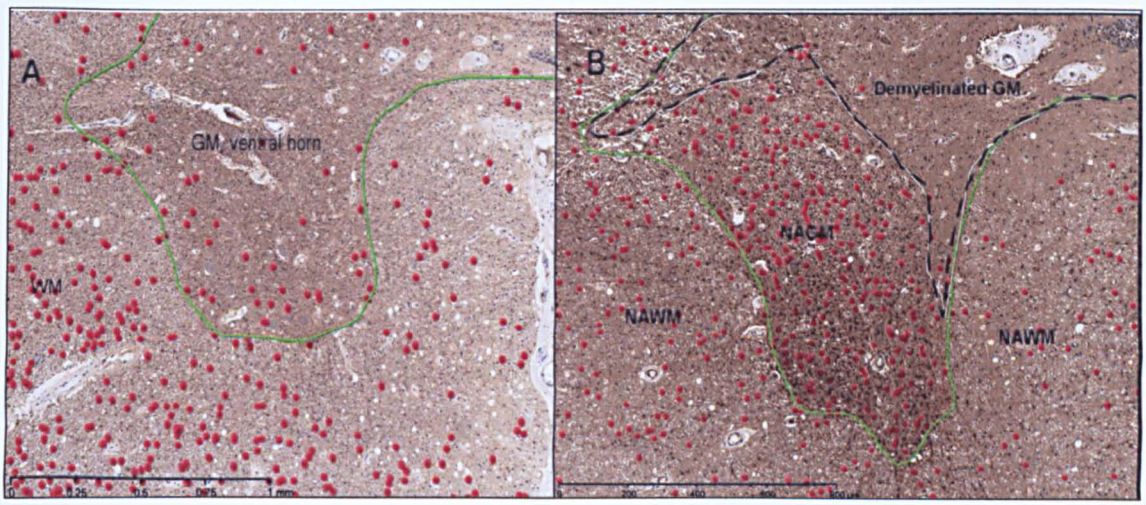


Figure 4.5: The image in A is the right VH of upper cervical segment from control subject. OLs are seen scattered in the GM and the WM. GM OLs are relatively fewer in the GM of controls compared to the WM (scale bar 1 mm). The image in B is right VH from MS subject. There is demyelinating lesion affecting part of the VH. Note clustering of OLs in the NAGM. Same lesion extends to the nearby WM with no obvious proliferation in the WM OLs (scale bar 0.8 mm).

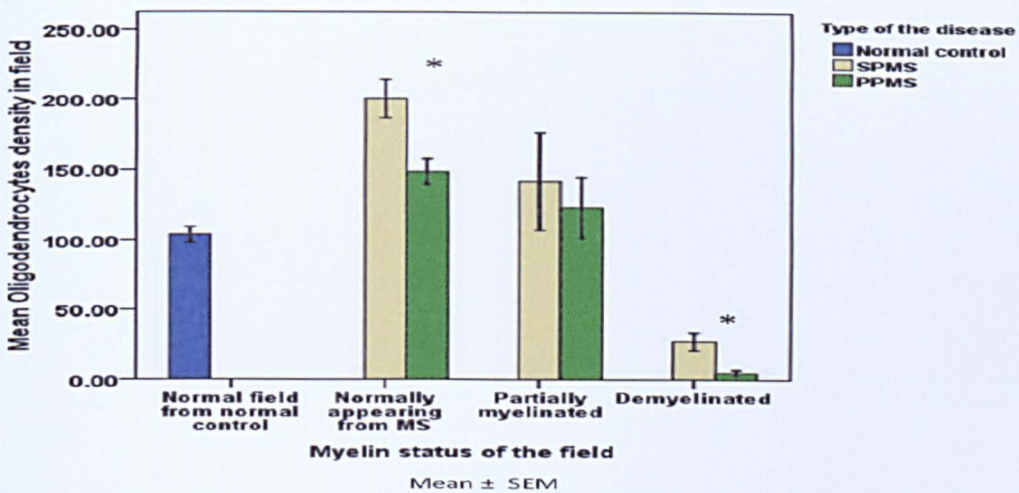


Figure 4.6: A bar chart demonstrating the variability in OLs density in the Normal controls and MS. Results include the VH and the DH. The bar heights represent the mean OL density in the GM ± SEM. Significant differences are indicated by an asterisk for p values <0.05.

Chapter 5: Pathology of oligodendrocytes in the WM of progressive MS spinal cords

5.1 Introduction

WM OLs or interfascicular OLs seem to have a vital role in the repairing mechanism of the CNS. Failure of the human CNS to compensate adequately for axonal and myelin loss is possibly the main cause of permanent disability after CNS injury. In CNS injury following MS lesions, OLs may produce some remyelination in the brain, but there is little information in relation to the extent, timing and clinical consequences of OLs' proliferation and the consequent remyelination in the spinal cord. This study will quantify OLs in the WM of the spinal cord in healthy controls and in the two progressive forms of MS. In this introduction, pathology of OLs and their role in remyelination in acute and chronic WM lesions will be reviewed.

5.1.1 Fate of oligodendrocytes in acute WM lesions

During the early stages of MS lesions, OLs pathology was found to be variable [261-267]. In some patients fresh lesions show preservation of OLs, while in others there is significant loss of OLs [261, 267]. Complete loss of OLs in the centre of the plaque has also been demonstrated [267]. This has been argued to be due to heterogeneity of MS lesions, raising the possibility that there is variability in OLs' pathology among different patients. Similar degrees of inflammation, axonal loss and OLs' loss were seen among different lesions from the same person [179, 267]. Lucchinetti et al described two main patterns of OLs' loss within the centre of the plaque compared to the active edge of the same plaque; in 70% of cases there was variable reduction in OLs' numbers in remyelinated or demyelinated lesions, while in 30% there was extensive destruction in OLs' numbers with no associated remyelination [179].

Causes of oligodendrocytes loss in acute lesions

OLs are amongst the most sensitive cells in the CNS, due to their high metabolic activity. In vitro studies show that OLs are very sensitive to immune mediated and cellular mediated damage [268].

Antibodies against OLs have been detected in the sera of MS patients [269, 270]. These antibodies have been detected in other neurological diseases and are thus non disease specific [270, 271]. Non specificity of CSF antibodies to MS disease and the lack of expression of MHC II expression on OLs suggest that OLs are not directly part of the presumed immune destruction [272]. Therefore, the role of these antibodies in causing damage to OLs is questionable [273, 274].

The immune attack of MS can cause early OLs' damage via T lymphocyte activation, especially via activation of CD8 cells. OLs were induced to express MHC I and II in vitro [275], but in vivo they express only MHC I [272, 276]. Although OLs were not observed to express MHC II molecule in vivo, CD4 cells can affect OLs' survival via different mechanisms. The cytotoxic CD4 cells can stimulate other cytotoxic cells, such as microglia and/or macrophages. OLs also express receptor for TNF-α, which can be secreted from CD4 cells [275]. TNF-α causes apoptosis of OLs in vivo and in vitro, but this effect needs high concentration and prolonged exposure [277]. Nevertheless, there is debate whether TNF has undesirable effects on OLs' survival and remyelination or not.

T lymphocytes also colocalised with expression of heat shock protein. Heat shock protein is incorporated in an immunologic reaction at the edge of lesions of MS, which may play a role in early OLs damage. Expression of heat shock protein-65 was found to be significant in OLs at the lesion's border, and colocalised with

gamma/delta T lymphocytes. OLs at the nearby normal areas did not show expression of heat shock protein [278].

Interaction of microglia and OLs was reported at the active border of MS lesions, but not in NAWM or inside the lesion. The adhesion molecule VCAM-1 is expressed by microglia. These microglia that are positive for VCAM-1 were distributed in the areas of active lesions and may target OLs, because considerable numbers of these cells were surrounding OLs [279]. In addition, although OLs' number correlates negatively with macrophage numbers [179], cytotoxicity of macrophages against OLs was found to be limited in MS [280]. Furthermore, there was no significant correlation with T cells or with axon density [179].

A recent mechanism was also suggested to induce apoptosis of OLs in chronic active lesions, which may play a role in acute lesions. OLs' expression of cyclooxygenase 2 (COX-2) was tested in MS and in experimental model of MS. COX-2 is an enzyme expressed by neurons that are susceptible to apoptosis. This enzyme is also expressed in OLs of chronic active lesions and in lesions of experimental model. OLs that express COX-2 were also found to express activated caspase 3, a marker of apoptosis [281].

The other proposed mechanism is that OLs may degenerate secondary to myelin loss. This is supported by the fact that OLs that fail to myelinate axons during development usually undergo apoptosis [194]. But it is not certain whether this mechanism of OLs' damage is responsible for acute or chronic loss.

5.1.2 Response of oligodendrocytes to acute WM lesions

There is debate about the fate of OLs in acute lesions of MS. Prineas and his colleagues argued that OLs are initially destroyed in active lesions, but then lesions are repopulated rapidly with new OLs [263, 282]. In comparison, Selmaj and colleagues reported preservation of OLs in acute lesions but then loss in chronic lesions [283]. Animal studies, however, support the first hypothesis. At day 14 post experimental contusion injury in rats, a dramatic increase in the formation of new OLs can be detected at the border of the lesion. In the spared regions there was increase in proliferation of NG2+ cells [226]. Timing of oligogenesis in animals is in agreement with timing of recovery from MS clinically (the recovery in the MS patient is usually within a few weeks) [226]. The importance of remyelination in protecting and restoring the conduction in axons has been confirmed previously in a viral model of MS [284]. In humans, remyelination is strongly believed to contribute, with other factors such as removal of oedema and inflammation, to recovery of patients' symptoms [43, 57, 66, 285].

Examination of the lesion at early stages of plaque evolution shows OLs associated with remyelination [263, 286]. Remyelination is characterised by thin myelin sheaths and short internodes [287]. About 23% of lesions were found completely remyelinated, forming shadow plaques [249]. Shadow plaques were described as early as 1906 [288]. Until the employment of the electron microscope in examining lesions of MS, shadow plaques were thought to be partially demyelinated plaques [289]. In the brains of MS patients 19% of lesions were partially remyelinated [249] and about 40% of lesions have some degree of remyelination that occupies more than 10% of the lesion area [282]. Nevertheless, in chronic lesions presence of OLs and remyelination fall dramatically. This decline in OLs and remyelination may be responsible for deterioration of symptoms.

5.1.3 Fate of oligodendrocytes in chronic WM lesions

In patients with disease duration that is more than 20 years, premyelinating OLs' numbers decline drastically [179, 236]. Although number of OLs reduces with progression of the disease, there are still considerable numbers of OLs in chronic lesions of MS [236, 237, 239].

OLs precursor cells' (O4 positive) density in spinal cord chronic lesions can reach up to 35/mm^2. A number of studies have demonstrated the presence of immature OLs in chronic MS lesions. These have been traced using O4 antibody [237], PDGF-α [245], and NG2 proteoglycan [244]. There was no difference in numbers of precursor cells between PPMS and SPMS [239]. A summary of previous publications that quantified OLs in chronic lesions of MS can be reviewed in Table 5.3.

A study carried out on lesions from the periventricular WM illustrated that chronic MS lesions contain significant numbers of OLs' precursors. These cells were positive for O4 but negative for mature OLs' markers. However, these cells were assumed to be quiescent (premyelinating), because there were no myelinated axons around them [237]. Premyelinating OLs are not normally found in the adult human brain, and are defined as cells with multiple processes that do not contact myelin internodes and are positive for O4 [236, 237].

5.2 Aims and hypothesis

The effect of spinal cord GM demyelination on the disease course is not yet established. When compared with poliomyelitis, which causes flaccid paralysis by destroying the motoneurons of the GM, MS causes predominantly spastic paralysis of the limbs, which supports to some degree that WM axonal pathology is the main cause of disability. Spinal cord pathology, including axonal loss and atrophy, was

found to correlate with disability better than brain pathology. Therefore, attempts to enhance remyelination of the WM in the spinal cord may produce better outcome. OLs' number and function are important for myelination and remyelination [236, 245]. The main aim from studying OLs in MS is to prevent their loss and/or promote their proliferation and differentiation to protect axons.

OLs in the WM are different from GM OLs by being in close proximity to axons. We have previous data about axonal loss in the spinal cord, but little data is available about OLs' pathology in the WM of the spinal cord. Both axons and OLs may affect each other. In this study, a comparison was made between OLs' numbers in the WM and the GM. Similar to the method used in the previous chapter, OLs were counted manually in selected fields from the CST and the PCP. Numbers of OLs were counted in the NAWM, demyelinated WM (DMWM) and the partially myelinated WM (PMWM). Numbers of axons were counted in the same areas of the same tracts in the next chapter. OLs numbers obtained in the CST and the PCP will be compared with number of axons in each field.

5.3 Material and methods

Except for the controls that were obtained from Nottingham, we used the same sample that has been used in Chapter 4. For this study, the same CA II-stained slides were used to count OLs in the WM. We have already analysed numbers of OLs in the GM. For the purpose of counting OLs in the WM of the spinal cord, we sampled two of the major descending and ascending tracts; the CST and the PCP. In all the digital slides, and using the NDP view and the digital pen, the boundaries of both the CST and the PCP were identified.

5.3.1 Sampling of the corticospinal and the posterior column pathways

Again, an unbiased sampling protocol was used to select fields of interest from the CST and the PCP irrespective of myelin status. The area of the CST was selected by referring to previous descriptions [122, 124]. For objective description; it is the area that is located in the lateral funiculus and dorsal to a horizontal line drawn through the dorsal border of the GM commissure. While the dorsal funiculus contains purely axons from the PCP, the lateral funiculus contains, in addition to the CST, fibres from other tracts such as the spinocerebellar tract. Each side of the CST was represented by 5 fields. Therefore, each section was represented by 10 fields. The sampling protocol was carried out as described in Figure 5.1. From the PCP, 5 fields were selected from each side of the PCP; therefore, 10 fields were exported from each section as shown in Figure 5.2. Similar sampling protocols of the CST [124] and the PCP [122] have been previously used in literature.

Similarly to the GM, the selected fields from the WM were exported as an image of the computer screen at magnification of 40X and screen resolution of 1024x768 (field surface area was 0.047 mm^2). When the WM content was very small, like in lumbar cords, fields were exported with smaller surface areas at magnification of 63X (surface area was 0.0164 mm^2). The majority of sections were exported at 40X magnification (95%).

5.3.2 Oligodendrocyte counting

Identification criteria of WM OLs are not different from the previously mentioned criteria of GM OLs in Chapters 3 and 4. We count OLs in the WM manually using the same method as previously described in Section 4.3.3.

Like in the GM, the myelin status of WM fields was identified retrospectively after counting OLs. The myelin status of each field was identified using the same method mentioned in Section 4.3.5. Fields were classified into DMWM, PMWM, and NAWM.

5.3.3 Statistics

Results were exported to SPSS analysis software. Student t-test was used for comparison of the mean. Both Bivariate correlation and partial correlation were used to study effects of age and disease duration. Multiple linear regression was used to study effects of age, disease duration, disease type, and myelin status on WM OLs density.

5.4 Results

From MS cases, about 58 WM lesions were examined (CST and PCP). From these, 1206 fields were exported (DMWM n = 267, PMWM n = 222, and NAWM n = 717). Numbers of fields and their myelin status is summarised in Table 5.1.

Appearance of CA II-stained OLs in the WM (interfascicular OLs) does not greatly differ from those in the GM. OLs appeared round to oval in shape with variable sizes and stain intensity. As in the GM, darker OLs in the WM tended to be smaller. In normal controls, distribution of WM OLs was not as homogeneous as in the GM. OLs were seen condensed at the entrance of the dorsal root. These OLs are believed to be type 4 OLs. It was also noticed that there was a higher number of OLs at the outer border of the WM (Figure 5.3).

Inside the WM lesions, OLs appeared isolated with no evidence of myelin around them. At the lesion border, OLs were observed to be intensely stained with CA II and were associated with scattered myelinated axons. Intensity of staining of both OLs

and myelin at the lesion border appeared to be greater than in the NAWM and normal controls (Figure 5.4).

Table 5.1: Number of analysed fields from the white matter

Type of the disease	Myelin status of the field (first time)	Number of CST fields	Number of PCP fields
Normal control	Normal controls	120	88
PPMS	Demyelinated	49	62
	Partially myelinated	46	44
	Normally appearing areas	213	206
SPMS	Demyelinated	72	84
	Partially myelinated	68	64
	Normally appearing areas	136	162

5.4.1 Quantification of oligodendrocytes in the WM

In healthy controls the average density of OLs in the WM of the two tracts was 98 ± 100/mm^2. This does not differ from OLs' density in healthy GM. In MS, regardless of myelin status or type of the disease, the average density of OLs in the WM was 72 ± 114/mm^2. This was lower than the calculated density in WM of normal controls ($p = 0.001$). In both MS and controls the density of OLs was greater in the CST compared to PCP. In the CST of controls the density was 116 ± 110/mm^2 and in the PCP the density was 73 ± 78/mm^2 ($p = 0.01$). Similarly, in MS OLs' density in the CST (mean 79 ± 126/mm^2) was greater than the PCP (65 ± 102/mm^2) ($p = 0.038$).

We also examined whether there was any variation in OLs' density among different cord segments. We found that the lower cord showed higher density of OLs compared to the upper cords in both controls and MS. One way ANOVA was used to examine for differences in OLs' density between the three main spinal cord levels. There was significant difference among groups in controls ($F(2\text{-}204) = 4.47$, $p = 0.03$) and in MS ($F(2\text{-}1203) = 30.00$, $P < 0.001$). In the previous chapter, we did not find a difference between GM OL density between various cord levels.

5.4.2 Oligodendrocytes' density in the NAWM

Analysis was further specified to include myelin status. The mean density of OLs in the NAWM of MS, including the two tracts, was found to be 104 ± 133/mm^2, which does not differ from the density calculated from WM of controls (98/mm^2). Within the MS group, OLs' density in NAWM was less than OLs' density in the NAGM, which was found to be 173 ± 161/mm^2 in the previous chapter ($p < .001$).

When the two disease types are compared, the mean density of OLs in the NAWM of SPMS was 69 ± 84/mm^2, while in the PPMS, the density was 130 ± 155/mm^2 ($p = 0.002$). Note that in the previous chapter the GM showed opposing results, where the NAGM of SPMS showed greater density in contrast to the NAGM of PPMS.

5.4.3 Oligodendrocytes' density in the PMWM

Density of OLs in the PMWM of the whole MS sample was 45 ± 60/mm^2. This number was significantly less than normal healthy controls and less than NAWM of MS ($P < 0.001$ for the two comparisons). Similar to the NAWM, which has less OLs' density than NAGM, the density in PMWM, was also less than PMGM areas (p value < 0.001). By comparing the PMWM in the two disease types, OLs' density in PPMS was 64 ± 69/mm^2 compared to 31 ± 48/mm^2 in SPMS ($p < .001$).

5.4.4 Oligodendrocytes density in the DMWM

The mean density of OLs in the DMWM was 7 ± 22/mm^2. In comparison, OLs' density in the DMGM was 17 ± 39/mm^2 ($p = 0.016$). There was no significant difference between PPMS and SPMS in the density of OLs in the DMWM. The density was 9.4 ± 27/mm^2 in SPMS subjects and 4 ± 13/mm^2 in PPMS subjects ($p = 0.602$) (Figure 5.5).

5.4.5 Effects of age and disease duration on WM oligodendrocytes

The effect of age on the WM OLs of controls was not significant in our sample (correlation = - 0.037, p value = 0.594), controlling for gender and cord level. In the MS sample, effect of disease duration was significant on the DMWM and NAWM regions, but not on partially myelinated areas. However, the correlation coefficient was too low for all the regions (Table 5.2). Similarly, results obtained from the GM showed significant effect of disease duration on the completely demyelinated areas.

Table 5.2: correlation between WM oligodendrocytes and disease duration

Myelin status of the field	Control Variables	Disease duration in years	
DMWM	Gender & Level of the cord & type of the disease & Age	Correlation	.138
		Significance	.027
PMWM	Gender & Level of the cord & type of the disease & Age	Correlation	.056
		Significance	.418
NAWM	Gender & Level of the cord & type of the disease & Age	Correlation	.075
		Significance	.047

5.4.6 Difference between CST and PCP in oligodendrocytes density

In MS cases, comparison was made between the two tracts based on the myelin status of the field. In NAWM, the mean density of OLs in the CST was 114 ± 149/mm^2 and in the PCP was 94 ± 116/mm^2 (p value 0.041). In the partially myelinated fields, the density was 38 ± 50/mm^2 in the CST and 51 ± 68/mm^2 in the PCP (p = 0.116). The demyelinated areas of the CST had a density of 13 ± 31/mm^2 while the demyelinated areas of the PCP had 2 ± 8/mm^2 density of OLs (p vale <0.001).

5.5 Summary of results

The average OLs' density in the WM of MS sample was less than normal controls. This reduction was due to loss of OLs in the DMWM with no difference between the NAWM and controls.

Regional variation in OLs' density was found between CST and the PCP and between different cord levels. There was significantly greater density of OLs in the lower cord levels in both controls and MS.

Unlike the GM, which showed greater density of OLs in the NAGM SPMS compared to PPMS, the NAWM showed greater density of OLs in PPMS compared to SPMS. However, there was greater density in the DMWM of SPMS compared to PPMS, but this was not statistically significant.

5.6 Discussion

This study is, to our knowledge, the first study that has investigated the difference in OLs' pathology in PPMS and SPMS in WM lesions of the spinal cord. The study also compared the GM and the WM using human spinal cord cross sections. We concluded from the results that OLs in the WM probably behave differently compared to GM OLs, where density of OLs in the NAWM and DMWM was less than NAGM and DMGM respectively. There is also variation in OLs' density between PPMS and SPMS; density of OLs was found to be greater in the NAWM and PMWM of PPMS compared to SPMS. The study has also demonstrated the presence of CA II positive OLs in chronic demyelinated WM lesions.

5.6.1 Variation between the grey matter and the white matter

In Chapter 4, we discussed the possible explanation of having greater density of OLs in the NAGM compared to control GM. In this chapter, there was no significant difference between the NAWM and healthy WM. In conclusion, we found that there is a greater number of OLs in the GM compared to the WM of MS. In the entire sample, the three examined areas; NAGM, PMGM, and DMGM have higher density of OLs compared to NAWM, PMWM, and DMWM respectively.

It is almost certain that the GM environment is different from that of the WM, or there are extra sources of OLs in the GM. The GM probably has an environment that it is more suitable for OLs' proliferation. It is well known that OLs' precursor cells are sensitive cells, and their proliferation is affected greatly by the surrounding environment. Proliferation of OLs is vigorously induced by PDGF, basic fibroblast growth factor 2 [290], and insulin-like growth factor 1 [291], In vitro studies demonstrated very sensitive precursor cells; adding fibroblast growth factor and PDGF to OLs' precursors caused marked increase in proliferation [292, 293]. Studying the degree of expression of these molecules may reveal differences between the GM and the WM.

The WM also has elements that inhibit OLs growth or maturation. Myelin is a potent inhibitor for OLs differentiation [294]. An in vitro study reported that myelin taken from demyelinated lesions (experimental lesions) significantly impaired remyelination by arresting OLs' proliferation. Consequently, limited OLs' proliferation in MS lesions has been attributed to depletion of macrophages which are essential in cleaning myelin debris [294].

Another factor that has been reported to affect OLs' survival is the presence of axons, which are more abundant in the WM. Abnormal axons may inhibit maturation of OLs. There is normally vital interaction between axons and OLs. The effect of axons on OLs is apparent in normal controls, where number of OLs is affected by density and diameters of surrounding axons [162, 167]. One OL can myelinate up to 50 small axons, but can myelinate smaller numbers of large axons [162]. In our study, OLs' density in the motor CST was found to be greater than the PCP in normal controls, which further supports the regional variation in OLs' density in the WM depending on axonal diameter (note that the diameter of axons in the CST is less than the PCP [295]).

Axonal signals are believed to be the main regulator of OLs' survival, proliferation and differentiation [296]. One of the signaling molecules derived from axons is neuregulin 1 [297]. Neuregulin is secreted from neurons and some astrocytes. Neuregulin expression was found to be reduced dramatically in active and chronic lesions of MS [298]. In an animal model of MS, neuregulin was found to be a potent stimulator of remyelination [299]. Since previous studies showed that the surviving demyelinated axons in MS are usually dystrophic [236], and that the level of neuregulin is reduced, differentiation of OLs may be impaired in the WM more than GM.

In addition, OLs in the WM are myelin maintaining cells. In the GM many OLs acquire perineuronal position and survive without essentially myelinating axons [160, 162]. Because we do not have neuronal tracts in the GM, axonal loss in the NAWM and DMWM may affect survival of OLs and further reduce their numbers. This may result in damage to OLs that lose their myelin processes. A previous study showed that numbers of demyelinated OLs, which are the mature OLs that lose contact with their

myelin internodes, reduce with chronological progression of the disease. They were found to be abundant in early lesions of MS (can reach up to 700/mm^2) [238]. There are no previous data about the correlation between axonal and OLs loss in MS. In the next chapter, axonal density and numbers will be quantified in the CST and the PCP. It is important to notice that we found greater loss of OLs in the WM of SPMS compared to PPMS. This may be secondary to loss of axons in these areas which has been reported to be greater in SPMS [72].

On the other hand, following injury there is an inhibitory effect of non-myelinating OLs and myelin on CNS axonal growth. For example, Nogo-A, MAG, and OLs myelin glycoprotein have been demonstrated as axonal growth inhibitors of the mammalian CNS [300]. In a study on mice, administration of antibodies, monoclonal antibody IN-1 generated against Nogo-A, has improved significantly regeneration and plasticity of the CNS [83]. Thus, OLs may have negative undesirable effects on axons, and as a result a vicious cycle of damage occurs to both elements. Therefore, future immunological studies should focus further on identifying the immunogenic characteristics of the WM and the GM.

Another explanation is that the GM possesses extra sources of OLs' progenitors compared to the WM. The human spinal cord GM is nearer to the central canal, which contains significant numbers of precursor cells. These are the nestin positive cells that has been discussed in Chapter 4 [260] Nestin is a marker of early neuroepithelial cells that may give rise to neurons or glia [301]. An important characteristic of these cells is that they are migratory [160]. It is possible that nestin positive cells proliferate around the central canal and then migrate to the nearby GM following injury. Migration of these cells probably does not reach the WM, as animal studies showed that OLs do not usually migrate long distances to myelinate axons

[302]. In MS specifically, it was observed that brain lesions proximal to the ventricular zone have a greater number of OLs [244]. We have also mentioned in Chapter 4 the possible role of perineuronal OLs in increasing numbers of GM OLs in response to injury. These cells are not present in the WM and may be responsible for the observed high numbers of OLs in the NAGM.

5.6.2 Nature of CA II positive oligodendrocytes

There is no previous study that has quantified OLs in MS using CA II stain. Most of the previous studies attempted to stain OLs progenitors using other markers, such as PDGF-α, NG2, A2B5, and O4. These markers do not stain all OLs lineage like CA II. In addition, some of these markers are not 100% specific for OLs progenitors. For example, A2B5 can be traced in some neurons and astrocytes and NG2 can be traced in microglia [277], while PDGF can be traced in some neurons [303, 304].

As we mentioned in Chapter 4, almost all the examined lesions were found to be chronic [138]. The present study revealed that the mean density of CA II positive OLs in chronic spinal cord WM lesions is 7/mm^2 (0-147/mm^2). Previous studies have examined the identity and role of OLs in chronically demyelinated WM lesions, and most of them found that these OLs are non-myelinating [236-239]. One study previously quantified OLs in chronic demyelinated lesions of the spinal cord and found that these cells are premyelinating or non myelinating [239]. Number of premyelinating OLs can reach up to 35/mm^2 in the spinal cord and 34/mm^2 in the brain WM lesions using O4 marker (O4 positive and GalC negative cells) [237, 239], and up to 40/mm^2 in brain lesions using NG2 marker [236]. The number reduces tremendously to 0-2/mm^2 for GalC positive cells [238]. GalC is the first marker to be expressed in differentiating OLs. Therefore, most researchers believe that these cells are quiescent and mainly represent premyelinating OLs that fail to mature to

myelinating OLs [237, 239]. Mature OLs that survived demyelination are frequent in early MS lesions but rare in chronic lesions, and become more restricted to the lesion border [238]. MOG positive OLs were also absent in silent lesions of MS [305]. Consequently, it is possible that most of the CA II positive cells inside lesions are immature OLs.

The maximum density of OLs in the demyelinated regions was 147/mm^2 in the present study. A recent study showed that density of OLs in the inactive lesions of MS can reach up to 250/mm^2 [179]. The mean duration of the disease in that study was 3 years, compared to 24 years in our study. A study carried out in 1994 counted OLs and degenerating OLs in chronic WM lesions of the brain. Density was 0/mm^2 for OLs and 7/mm^2 for degenerating OLs [306]. The CA II marker used in our study possibly stains surviving and degenerating OLs. Other studies are also summarised in Table 5.3.

In conclusion, CA II cells inside lesions are likely to be immature or quiescent cells. This is supported by the observation that presence of these cells was not associated with WM or GM remyelination. Future studies may double label CA II stain with MOG or O4 to identify these cells. For example, CA II positive and MOG negative are most probably newly formed immature OLs, while CA II positive and MOG positive are mature OLs that have survived demyelination [238].

In the lesion border, cellular composition is variable since the border may be active even in the chronic lesions. A previous study calculated the density of OLs in the lesion border of brain WM and found it to be 19 OLs/mm^2 and 14 degenerating OLs/mm^2 (sum = 33/mm^2). The mean density in the present study was 45/mm^2. The lesion border may contain immature OLs or OLs that survived demyelination [238].

The observed variability in OL density between studies can be attributed to the following reasons; each study has different criteria of selection, different mean disease duration, and different markers used to trace OLs. In addition, acute lesions that affect early MS may produce a different pathological picture from acute lesions that affect chronic MS [306]. Numbers of OLs after any injury can be affected by the number of OLs in the area before injury, age of the patient, and proximity of the lesion to the ventricular zone [244].

5.6.3 Oligodendrocytes in chronic lesions and their role in remyelination

A number of studies have shown that remyelination occurs mainly in the acute or relapsing remitting phases of MS, while progressive MS is associated with a much lesser degree of remyelination [179, 238, 282]. Effect of disease duration on OLs' number was found to be variable. It is generally accepted that there is OLs loss in MS lesions with disease progression [239]. In another study there was no significant negative correlation [179], while in a subset of patients a positive correlation was found between remyelination and OLs density with time [307]. The present study has demonstrated very weak positive correlation coefficient between OLs' density in the NAWM and DMWM and disease duration, controlling for age, gender, cord level, post-mortem delay, and subtype of the disease. However, when the three areas are included in the analysis, MS showed significant negative correlation with disease duration. This may imply that the total number of OLs in MS is, in fact, decreasing.

New OLs in the lesions of MS were found to be positive to CA II [263]. However, it is already established that remyelination in MS is inadequate in most of the patients, and chronic lesions frequently demonstrate little remyelination [239]. In this study, remyelination within chronic WM lesions of the spinal cord was not observed. CA II positive cells were seen isolated without evidence of surrounding myelin. In

comparison, EAE frequently shows complete recovery even with chronic disease [172, 173, 239, 308, 309]. It is not well known why remyelination fails in human nervous system or why the number of premyelinating OLs in chronic lesions of MS reduces with disease progression [238, 239].

Most studies have demonstrated what is claimed to be considerable numbers of OLs in chronic lesions of MS (Table 5.3). However, we do not have information about the importance of these numbers and their adequacy to produce effective remyelination. In normal circumstances one OL can myelinate up to 50 axons, but in response to demyelination, one OL may remyelinate a different number. Remyelination is characterised by shorter internodes compared to normal myelination; therefore, higher numbers of OLs may be needed for remyelination of axons.

Moreover, enhancement of OLs' proliferation or transplanting OLs within chronic demyelinated lesions may not produce remyelination [248]. A number of studies showed that OLs in chronic lesions of MS were not able to remyelinate axons [236-239, 245, 306]. Some researchers attributed limited remyelination in MS to failure of OLs' differentiation into mature myelinating OLs [236-239], while others suggest that axons are damaged and thus non receptive to being remyelinated [236]. Chronic lesions were found to be associated mainly with dystrophic axons with multiple swellings. Possibly, a combination of the two factors plays a role, in that a signal from dystrophic axons prevents maturation of OLs.

However, another study proposed that axons are not the cause of failure of remyelination. The study assumed that failure of remyelination is due to lack of inflammation in chronic lesions, since inflammation is believed to be a strong stimulator of remyelination [248]. The relation between inflammation, remyelination,

and OLs' pathology is probably more complex, and seems to be variable between the GM and the WM. A study reported that recurrent inflammation may inhibit remyelination, which may explain why the GM undergoes a greater degree of remyelination [310]. On the opposite side, another experimental animal study reported increase in remyelination of the WM after experimental induction of inflammation [248]. Although previous studies demonstrated little inflammation of the brain GM compared to the WM [311], the spinal cord GM may behave differently.

It has been also proposed that there are not enough precursor cells to produce OLs [255, 312]. A relatively recent animal study showed that prolonged treatment with glucocorticoids may inhibit proliferation and differentiation of OLs [313]. Another study reported interaction between astrocytes and OLs in the early stages of plaque development. Those OLs that interact with astrocytes were found to be immature and premyelinating. Astrocytes were therefore assumed to be partially responsible for failure of remyelination by causing apoptosis of OLs [314].

Variability between studies in evaluation of remyelination degree may be due to misinterpretation of other pathologies, such as Wallerian degeneration or acute partially demyelinated lesions as remyelinated lesions. Homogenous lesion with reduced density of axons and thin irregular myelin is the light microscopy picture of the remyelinated plaque. Reduced density of axons with normal thickness of myelin is the picture of Wallerian degeneration, while reduced axons with hypercellular lesion is the picture of acute partially demyelinated lesion [287].

In conclusion, it seems that both OLs and remyelination decline in progressive MS. Consequently, the time window for remyelination enhancement may be limited to the

first 15-20 years of the disease, especially in the relapsing remitting phase of SPMS, after which remyelination and OLs' numbers fall dramatically [236].

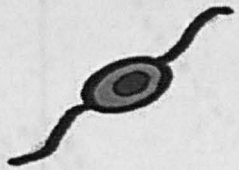
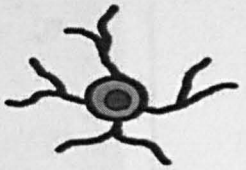
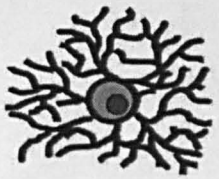
Table 5.3: Studies that have quantified oligodendrocytes in chronic lesions of MS

Study	Materials and methods	Mean duration of disease	Target cells and the used Marker	Density of OLs	Conclusion of the study
Lucchinetti 1999 [179]	Brain autopsies and biopsies	3 years	PLP, CNPase, anti MOG	Ratio of density in DMWM to NAWM range between 8% - 79%	Strong correlation between remyelination and OLs density Density of OLs in lesions of early MS is variable
Ozawa 1994 [306]	Brain autopsy	1-20	PLP mRNA, DNA fragments	60% of chronic lesions showed density of OLs <10/mm^2	In lesions of early MS, OLs are preserved, but in lesions develop later in MS, OLs are extensively destructed.
Wilswijk 1998 [237]	Chronic Brain lesions (around ventricles)		O4, GalC	4-34/mm^2	OLs in chronic lesions fail to differentiate into mature cells
Chang 2002 [236]	Chronic Brain lesions (post-mortem)	less than 20 years duration and more than 20 years	OLs progenitors and premyelinating OLs. PLP, MOG, NG2	25/mm^2	Premyelinating OLs are present in chronic lesions of MS. Cause of remyelination failure is unreceptive axons
Wilswijk 2002 [239]	Spinal cords lesions from 16 subjects	23.9 years	O4 and GalC.	35/mm^2	Negative correlation between age of lesion and O4 cells.
Scolding 1998 [245]	8 post-mortem brains and spinal cords	12 years	A2B5, GalC, and PDGF-alpha targeting OLs progenitors	1-3 OLs/100 nuclei (ratio of PDGF-α positive cells to the total number of nuclei in the field)	Demonstrated the presence of OLs progenitors in the human adult brain and lesions of MS (Both acute lesions and chronic lesions)

5.7 Conclusion

This study provided data about numbers of OLs in the spinal cords of patients with chronic progressive MS. A CA II positive cell may be immature OL, OL progenitor, mature OL or degenerating OL. There is a difference between GM and WM lesions in OLs' density. Further work should focus on identifying level of expression of certain molecules in the GM and the WM, such as PDGFR, insulin-like growth factor, and fibroblast growth factor 2. The study has further confirmed the presence of OLs in chronic lesions of MS. There was a difference in OLs pathologies among the two disease subtypes in the GM and the WM. For further understanding of this difference, double labelling of CA II positive OLs is essential, as CA II stains both mature and immature OLs.

Table 5.4: stage of OL maturation

Morphology					
Name	Pre-progenitors [288], precursors [160] or neural stem cells	Progenitors [288]	Embryonic Pre-Oligodendrocytes [288] or pro-Oligodendrocytes. In adults CNS, they are called NG2	Immature or premyelinating oligodendrocytes	Mature myelinating
Marker	Nestin, PDGF-α	A2B5, PDGF, NG2	O4, NG2, PDGF-α	O4, CNPase, GalC	O4, CNPase, GalC, MBP, MAG,PLP, MOG
Potential	Can give rise to neurons and glia [260].	Can give rise to OLs and astrocytes in culture	Some cells continue in the lineage to acquire myelination function, while others continue as the adult progenitor cells (NG2 positive) [288]	These cells are committed to give myelinating OLs [160]	Mature myelinating cells
Presence in normal adult brains	Has been recently identify around the adult human central canal, near ependymal cells [260]	Small numbers of PDGF-α positive cells have been identified in human brains, in normal healthy controls and in MS lesions [245].	NG2 positive cells or the well known OPCs, they are widely spread cells in adult CNS [244]	No evidence of their presence in adult human CNS in normal circumstances	Heterogeneous in shape and size and distributed in the GM and the WM
Presence in MS lesions	Not identified in lesions of MS	Small numbers has been identified in acute and chronic lesions [245].	NG2 positive cells have been identified in MS lesions and normally appearing areas. But their number is reduced in chronic lesions [244]	These cells have been identified consistently in chronic lesions of MS [236-239]	Few numbers of mature myelinating OLs have been identified in chronic lesions. More numbers of mature MOG positive OLs have been identified in acute lesions of MS [261, 287]). They mostly represent mature cells that survived demyelination [238]
Notes	These stem cells express Nestin and sometimes PDGF-α and maintain same morphology. But researchers believe that once they express PDGF-α, they become committed to OLs cell lineage. These cells are migratory and proliferative [160, 288]	These cells are bipolar or non-processed. They are believed to be the most efficient remyelinating cells in animal models. exogenous transplant of these cells resulted in remyelination of cultures axons [245]. These cells are migratory and proliferative [288]	These cells are proliferative but not migratory. Can differentiate in to OLs in culture. They are PDGF- α positive, but they are stellate in shape [244].	Short life cells; undergo apoptosis if they don't myelinate [194]. They are neither migratory nor proliferative [315]. They Have been identified in chronic lesions of MS in the brain and the spinal cord , but they fail to mature further and myelinate axons and their number reduces with disease progression [236]	Postmitotic. Many believe that they have no role in remyelination.

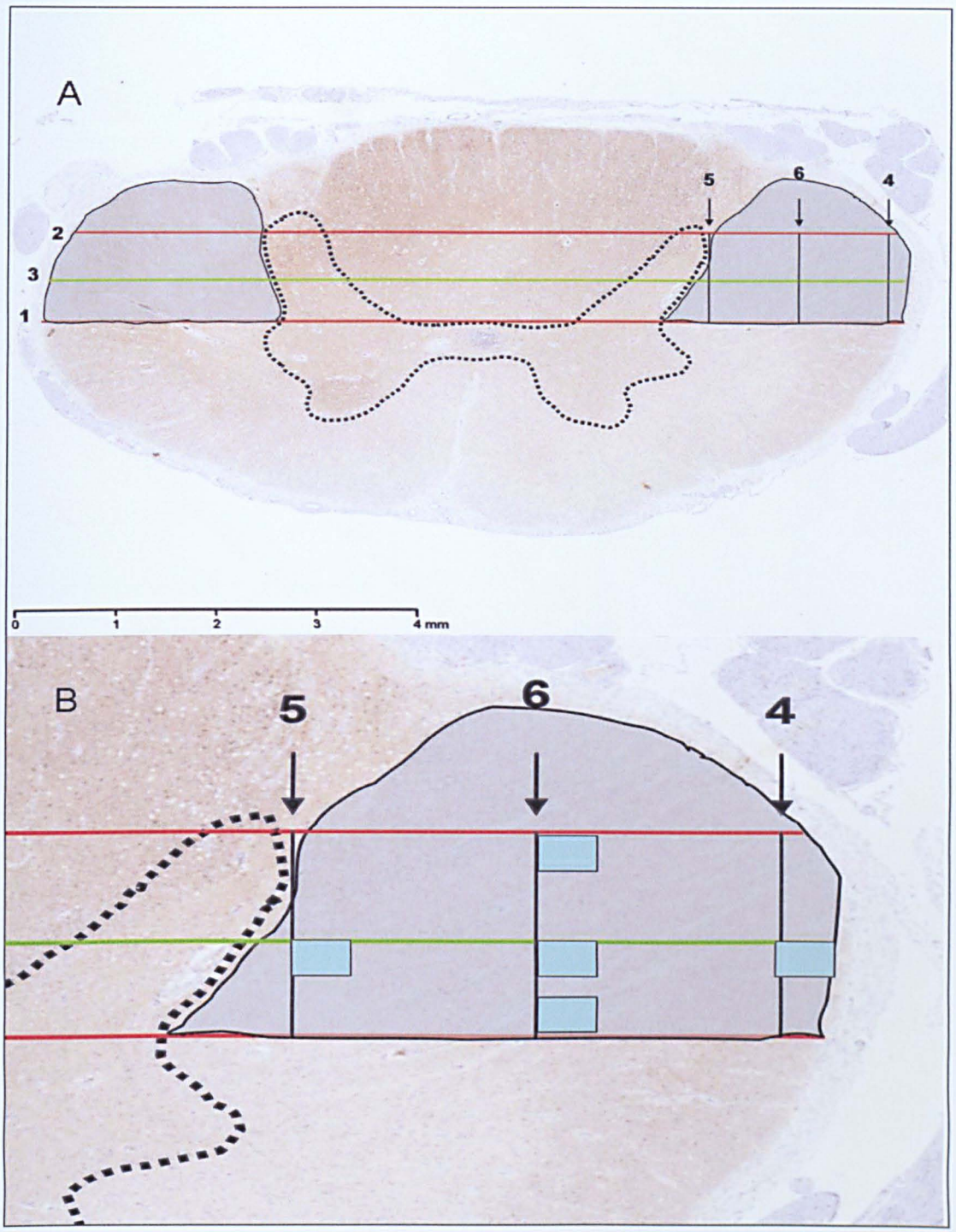


Figure 5.1: The sampling protocol of the CST. In A, a horizontal line is drawn through the spinal cord section. The line touches the dorsal boundary of the GM commissure (line 1). A second horizontal line is drawn at the midway between line 1 and the dorsal boundary of the PCP (line 2). The third horizontal line is drawn in the mid-distance between the two previous lines (line 3). The most medial intersection of line 1 or 2 with the CST boundary is identified and a perpendicular line is demarcated (line 4). The most lateral intersection of line 1 or 2 with the GM boundary is identified and a perpendicular line is drawn (line 5). The sixth perpendicular line is drawn in the mid-distance between lines 4 and 5. In B, Five fields were then selected.

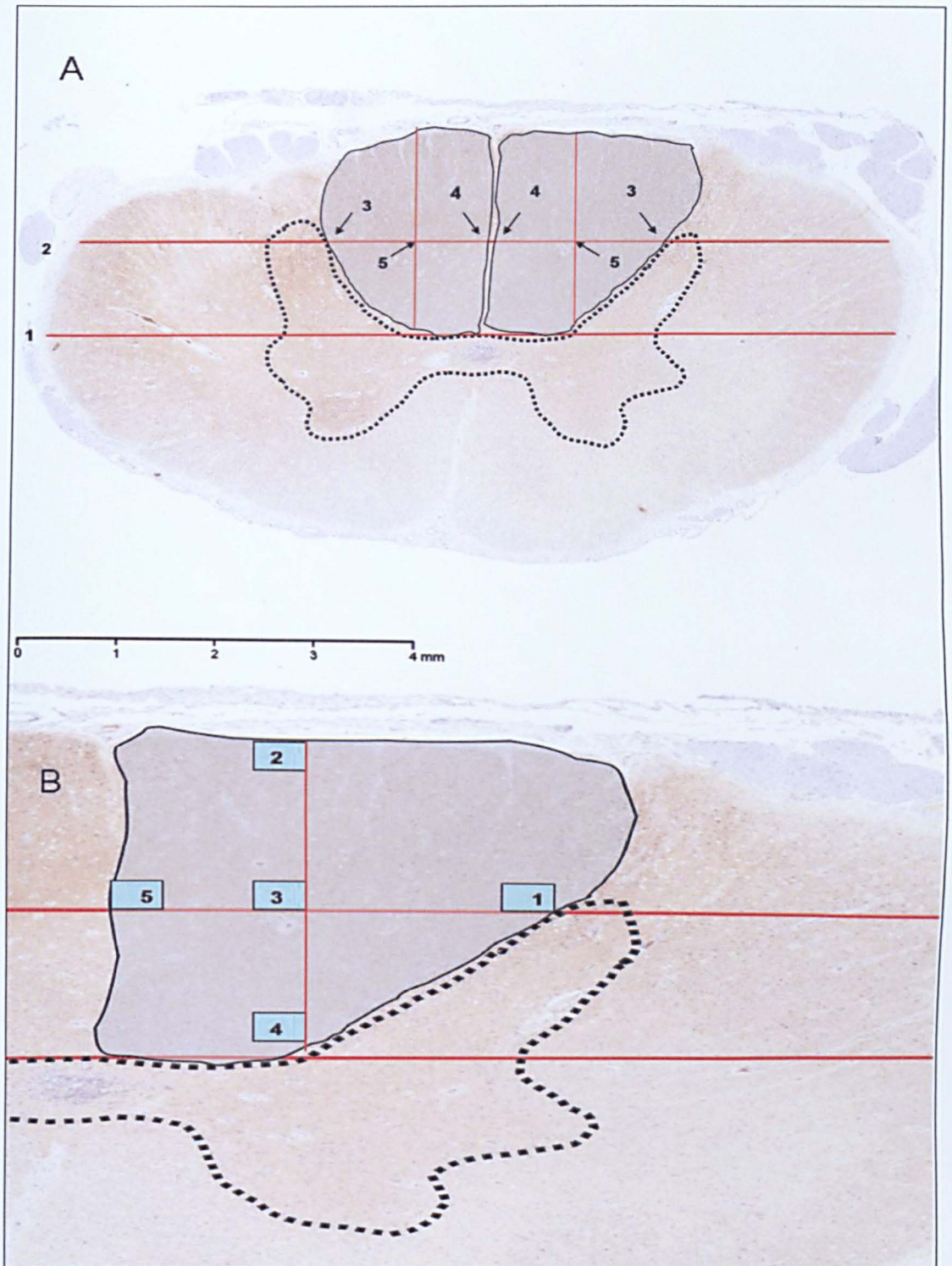


Figure 5.2: The sampling protocol of the PCP. In A, The first line is a horizontal line through the dorsal boundary of the GM commissure (line 1). The second line is parallel to the first one and lies in the mid-distance between the first line and the dorsal boundary of the PCP (line 2). The intersection of the second line and the DHs is identified (point 3). The intersections of the second line and the PCP boundary are identified (point 4). The midpoint between the two intersections 3 and 4 is identified and a perpendicular line is drawn through that midpoint (line 5). In B, 5 fields were exported from each side as shown.

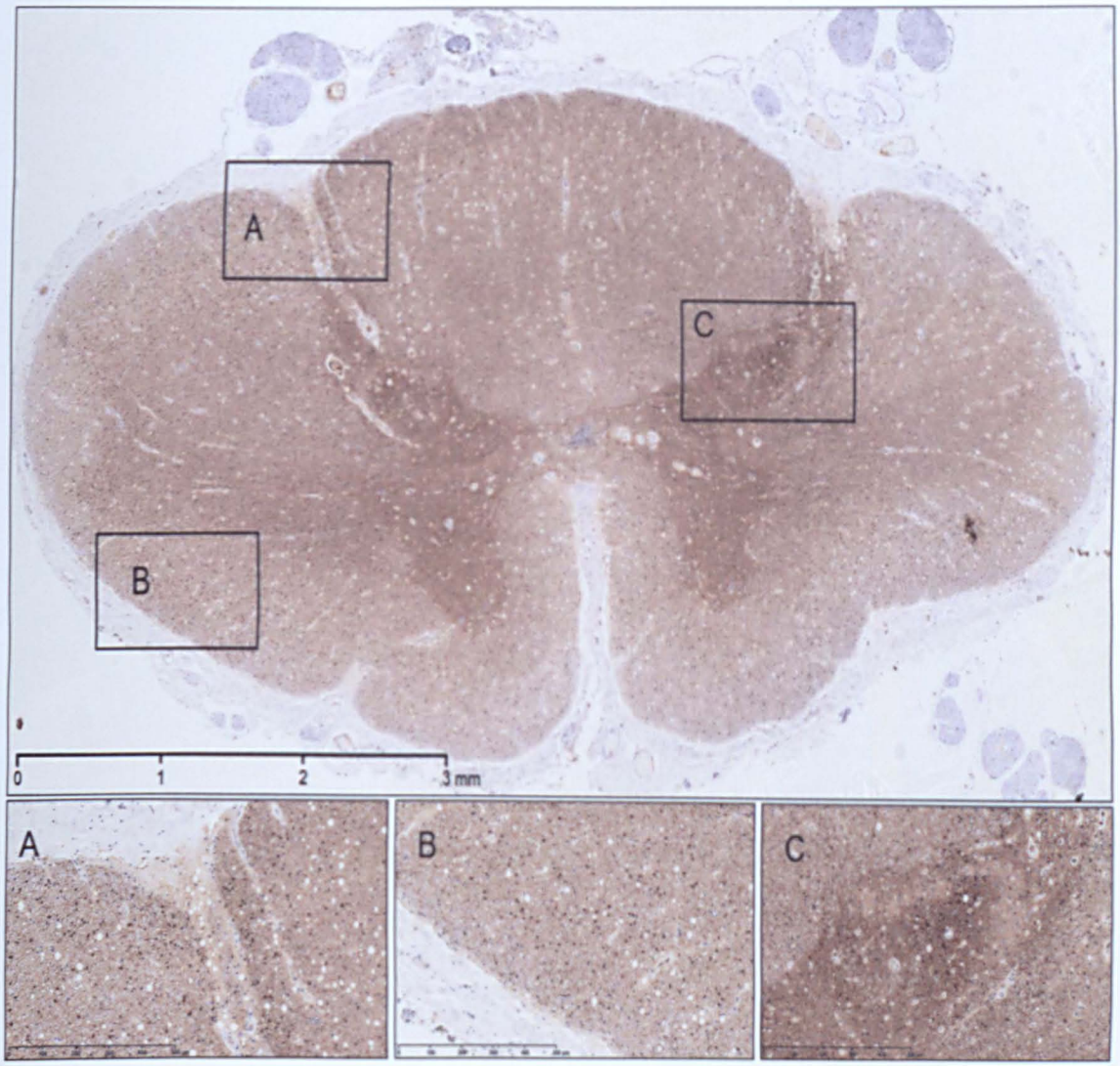


Figure 5.3 shows areas with accumulation of OLs (scale bar 3mm). Distribution of OLs around the entrance of the dorsal nerve root in A (scale bar 0.5 mm), at the outer border of the WM in B (scale bar 0.5 mm), and in the nucleus proprius of the GM in C (scale bar 0.5 mm).

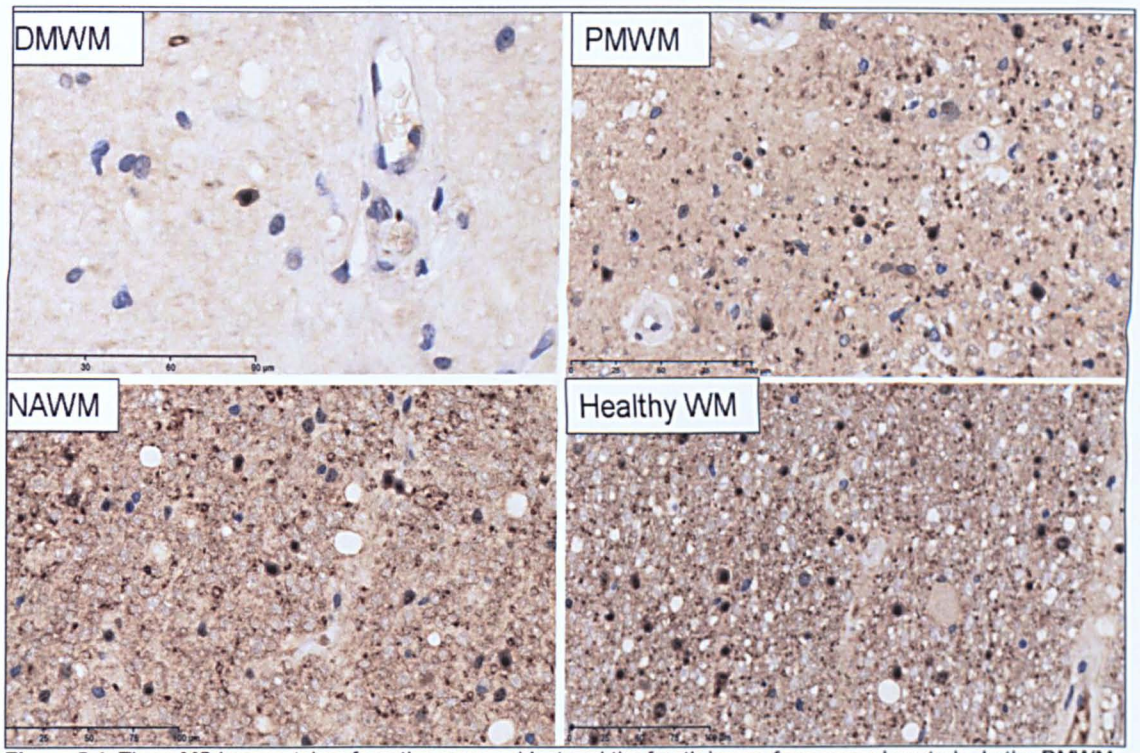


Figure 5.4: Three MS images taken from the same subject and the fourth image from normal controls. In the DMWM, OLs appeared isolated without evidence of myelin around them (scale bar 90 µm). Most of the previous studies have found that OLs within chronic WM lesions are not associated with myelin. At the lesion border (PMWM), OLs were associated with sparse but intensely stained myelin which may represent newly formed myelin as CA II is expressed intensely in myelinating OLs (scale bar 100 µm). In the NAWM and healthy controls, OLs are widely spread in between lightly-stained myelinated axons (scale bar 100 µm).

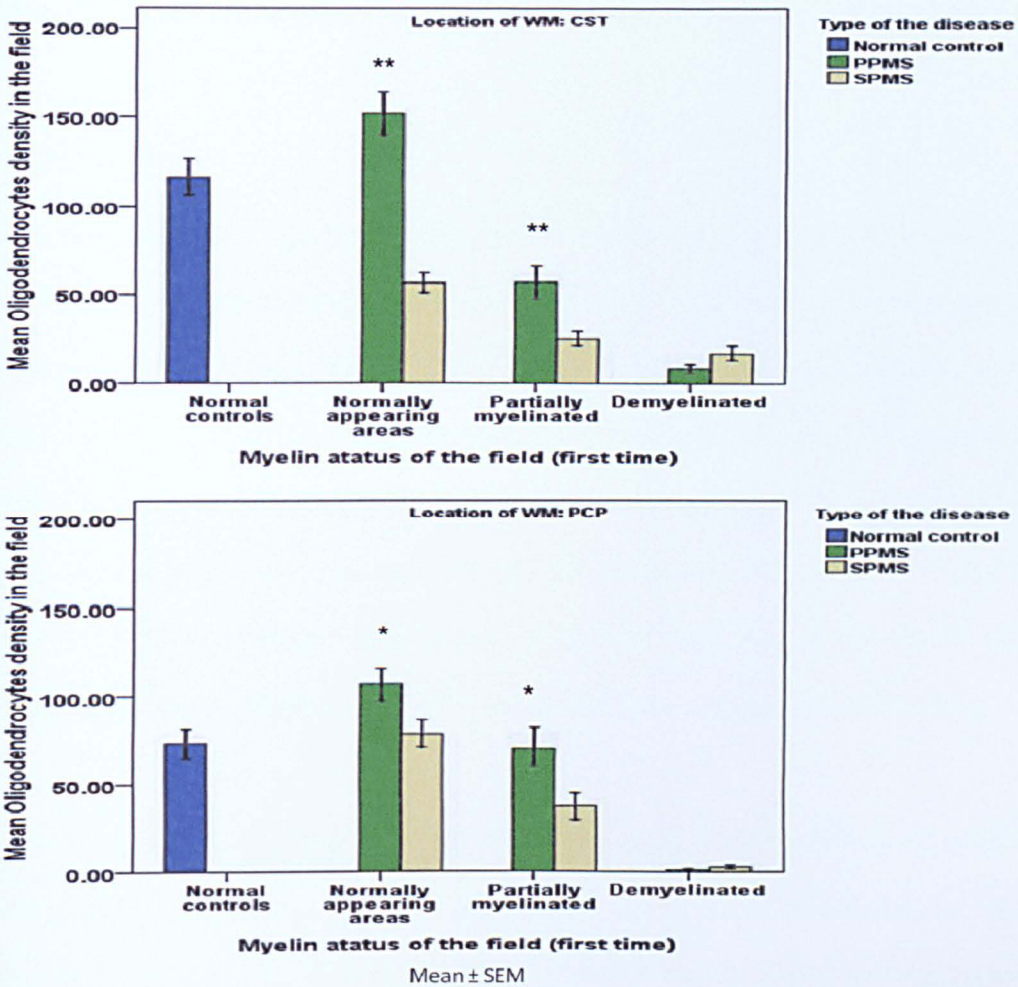


Figure 5.5: A bar chart showing OL density in normal controls and in the three examined areas in MS. The bars represent the mean OL density ± SEM. Significant differences of p values < 0.05 are indicated by one asterisk and p values of < 0.01 are indicated by two asterisks.

Chapter 6: Axonal loss in the spinal cords of PPMS and SPMS

6.1 Introduction

Axonal damage in MS has been recognised for many years, and was even reported when Charcot recognised the disease for the first time. Axonal loss is believed to be the cause of irreversible disability [316, 317], and has been correlated with disability better than other pathological or MRI findings [127, 318]. Axonal loss appears to be responsible for disability from the early stages of MS [243]. Two longitudinal MRS studies on the brain have shown that axonal damage correlates with disability in RRMS [319, 320]. Another longitudinal study has also reported that cumulative axonal loss is responsible for worsening of symptoms in progressive MS [321].

Axonal loss can be assessed in vitro by post-mortem histopathology studies or in vivo by estimating levels of NAA expression using MRS. Reliability of NAA in reflecting axonal loss has been validated by a post-mortem study, where a significant correlation was found between axonal loss and levels of NAA [127].

6.1.1 Early axonal loss

In EAE, the animal model of MS, axonal loss took place even before myelin loss [322]. A histopathology study has also reported significant axonal damage in the NAWM of a patient who died from acute MS [69]. Using proton MRS, significant decline in NAA detection was reported in the NAWM of the brain of early MS [243]. Acute axonal loss, labelled by Amyloid precursor protein (APP), was found to be extensive in the first year of disease evolution and reduces with time [323].

It is possible that early axonal loss is due to inflammation. Early in the plaque formation of MS, axonal loss accompanies demyelination and inflammation [324].

Ferguson and colleagues have confirmed transection of axons within acute MS lesions. The numbers of severed axons, labelled by APP, which is a sensitive marker of axonal damage, correlated significantly with inflammation [324] and with disease activity [325]. In acute lesions of MS, there was a significantly greater number of acutely damaged axons compared to chronic lesions [326]. These data support the role of inflammation in inducing axonal damage inside acute lesions.

On the other hand, a few other studies reported that axonal loss does not correlate with disease activity. A longitudinal MRI study followed 43 patients with RRMS for two years. The study assessed levels of NAA in the NAWM of the brain stem. The basal NAA detection was significantly less than controls. This continues to reduce during the follow up period with or without evidence of relapses [327]. In addition, NAA detection was significantly reduced in the cerebrum of MS patients in the early stages of the disease with low lesion load [328]. Similarly, a histopathologic study also revealed insignificant correlation between lesion load and axonal density [329].

Acute axonal loss can be induced by molecules released in the plaque [316] or that may diffuse outside the plaque to affect normally-appearing regions [242], such as Calpain or nitric oxide [316]. Adding CSF from patients with aggressive MS induced neuronal and axonal injury in culture, while CSF from patients with benign MS did not cause damage to the culture [330]. Glutamate toxicity may also cause neuronal and axonal damage early in MS [331].

6.1.2 Chronic axonal loss

Axonal loss continues to be a feature of chronic MS lesions and the NAWM. Both in vivo and in vitro studies have shown significant axonal damage in chronic lesions. For example, NAA levels were reduced in the normal appearing areas by 30% and

42% in demyelinated areas [127]. Bjartmar and colleagues calculated axonal loss in the spinal cords of chronic MS patients (via MRS) and reported 68% loss of axons within the plaque [69]. Another MRS study reported 32% axonal loss in the cervical segment of chronic MS [332]. A post-mortem study assessed axonal density in the cervical cord and found a 41% fall in axonal density [318]. In chronic MS, a number of studies have observed axonal loss in the NAWM of the corpus callosum [242], the CST [122, 318, 329], and the PCP [122].

It has been hypothesised that loss of axons in chronic and inactive lesions may be secondary to loss of the myelinating OLs within the plaque area [179]. However, degree and timing of axonal loss secondary to OLs' loss is not clear and some believe that OLs are lost secondary to axonal pathology.

Long term effects of neuronal degeneration may also result in loss of axons within the plaque and in normally appearing areas. This may include Wallerian, retrograde or anterograde transynaptic degeneration. In addition, the recently established mitochondrial theory suggests that mitochondrial pathology may play a role in axonal degeneration [333]. The mitochondrial respiratory chain complex 4 activity was reduced in lesions of MS [334] (for review see Su et al 2009).

6.1.3 Variation between PPMS and SPMS in axonal density

It was shown in Chapter 2 that SPMS spinal cords have greater atrophy compared to PPMS, and both disease subtypes have less atrophy in the caudal parts of the spinal cord. The significant contribution of axonal loss in atrophy was revealed previously [318], which may explain why atrophy and axonal loss are the only parameters that correlate with disability [127, 318]. This leads to the conclusion that atrophy may exert its effect on disability primarily via axonal loss or myelin loss. However, axonal

loss in various levels of the spinal cord has not been examined separately in PPMS and SPMS.

A study by Ganter in 1999 estimated axonal density and surface area of the spinal cord lateral column. The sample examined post-mortem spinal cord segments from C3 to T2. The segments were taken from MS subjects who lacked MS plaques at these levels. The CST surface area and axon density were significantly reduced in that study [318]. Only one study has examined for segmental variation in axonal pathology in MS spinal cords. However, the study did not differentiate between PPMS and SPMS. The study was carried out in 2004 and compared axonal loss between motor and sensory tracts in multiple levels of the spinal cord. There was significant decline in the CST area and axon density in all spinal levels. In comparison, the PCP showed reduction of the area and axons density only in the upper cord segments [122].

There are two previous pathological studies that compared axonal loss in PPMS and SPMS. The study by Bitsch in 2000 used brain biopsies from patients with MS and studied acute axonal loss by APP staining. The study demonstrated greater loss of axons in acute lesions of SPMS compared to PPMS [72].

The second study was carried out in our lab and analysed axonal loss in the CST of the cervical cord. Tallantyre et al (2009) differentiated between PPMS and SPMS and studied the axonal density and total number of axons in the CST. The total number of axons in the CST was found to be significantly reduced in the two disease subtypes compared to controls. In the demyelinated regions of the CST and compared to the NAWM, PPMS demonstrated more reduction of axonal density than SPMS [124].

For this chapter, tissue from PPMS and SPMS spinal cords taken from multiple levels will be analysed. The possible difference between motor and sensory neuronal tracts will be addressed. In this study we also compared axonal loss in three different areas; DMWM, PMWM, and NAWM.

6.1.4 Anatomy of the CST and the PCP

In this study, two tracts have been sampled, which are the CST and the PCP. These are the main tracts of the motor and sensory systems. They are easily demarcated as the PCP is localised to the dorsal funiculus and the CST is localised to the lateral part of the spinal cord (lateral funiculus).

The dorsal column-medial lemniscal pathway

The PCP or the dorsal column-medial lemniscal pathway is the main ascending tract of the spinal cord. It transmits mainly fine touch, conscious proprioception and vibration. Recently, the tract was found to have a role in visceral nociception [335]. PCP transection in cats causes marked dysmetria regardless of visual guidance of the limb [336]. In monkeys, transection of the PCP results in failure of discrimination of frequency and repetition of tactile stimuli [337]. In humans, ataxia can affect up to 80% of MS patients during the disease course, which can be due to lesions of the PCP or the proprioceptive mechanisms [338].

The first order neuron of the tract is found in the dorsal root ganglion. It receives input from receptors in the skin or deep stretch receptors in the muscle spindles and joint capsules. The tract ascends in the posterior funiculus of the spinal cord, where fibres from the lower body run medially in the gracile tract and fibres from the upper body run laterally in the cuneate tract. At the level of medulla oblongata, the two tracts synapse in nucleus gracilis and nucleus cuneatus [339]. However, only 25% of the

PCP axons reach the dorsal column nuclei in the medulla. Most of the axons, especially proprioceptive axons, leave the tract continuously to synapse in Clark's nucleus or nucleus proprius, which in turn continues as the spinocerebellar tract to mediate unconscious proprioception. The spinocerebellar tract continues ipsilaterally to synapse in the cerebellum. The second order neurons of the PCP run from the medulla oblongata and decussate to form the medial lemniscal pathway. The medial lemniscal pathway synapses in the thalamus. The third order neurons run from the thalamus and terminate in the primary sensory cortex to mediate conscious proprioception [339].

The corticospinal tract

The CST fibres originate from lamina V of the cerebral cortex and the brain stem and have connections with rostral and frontal parts of the cerebral cortex. Fibres of the CST are not completely myelinated until the age of 2 years. Axons of the CST synapse in interneurons of the VH or in sensory neurons of the DH. The average number of axons in the CST is 1 million in each side [340] (Figure 6.1). In adults, about 70% of the axons are myelinated and the diameter for the majority of axons ranges between 1 μm and 4 μm [339].

6.2 Aims and hypothesis

Maintaining axonal function seems to be crucial in determining disability in MS. The mechanism of axonal damage is not well understood. Patients with PPMS and SPMS have variable clinical course, but there are not enough data to suggest a different pattern of axonal loss that may explain this variation in clinical course. On the other hand, it is important to determine if accumulative axonal loss is mainly due to inflammation, and thus affected by relapses, or due to other degenerative processes. Studying axonal loss in various regions of the CNS in both ascending and

descending tracts may help in understanding the mechanism of damage. Especially because segmental difference in axonal loss between ascending and descending tracts of the spinal cord has been reported previously [122]. Axons are relatively spared in MS lesions when compared with loss of other elements such as myelin and OLs, but there is no previous study that quantified OLs: axon ratio.

In this study, axons will be counted in the CST and the PCP in multiple levels of the spinal cord. The study will differentiate the extent of axonal loss between PPMS and SPMS. The same sampling protocols of the CST and the PCP that have been used in Chapter 5 will be used in this chapter. Therefore, results of OLs numbers obtained in the previous chapter will be used to calculate OLs: axons ratio.

6.3 Material and methods

We used formalin-fixed paraffin-embedded sections from the same sample as in Chapter 5. Sources of tissue include the MS Society tissue bank (PPMS n = 13, SPMS n = 15, controls n =5) and Oxford Radcliffe NHS Trust (controls n = 6). Dissection and initial preparation is mentioned in Sections 2.5.1 and 2.5.3. As we have already mentioned, 10 sections were cut at 5 µm thickness from each block. We have already selected one section for MBP staining to study atrophy and demyelination in Chapter 2 and another section for CA II staining to study OLs in the GM and the WM in Chapters 4 and 5. In this study, another section was selected to trace axons in the WM by using anti-neurofilament (NE14). This will be used to count axons in the CST and the PCP. (Neurofilament 200 KD, NE14, and dilution 1:20,000, Chemicon). Four controls from the Oxford tissue were excluded due to inadequate staining (subjects B272, B458, B3269, and B3313 in Tables 2.1 and 2.2).

The NE14-stained slides were numbered so that the observer is blinded to the disease subtype. Slides were then converted to digital slides using the NDP.view Hamamatsu and viewed on the computer screen as previously described.

6.3.1 Sampling the CST and the PCP

The GM and WM boundaries were demarcated on NE14-stained slides. Then areas of the PCP and the CST were also identified as in the previous chapter. The surface areas of the CST and the PCP were measured.

We have already established a protocol to sample the CST and the PCP to quantify OLs. In this study we have applied the same sampling protocol on NE14 sections to quantify axons in the same fields. This enabled us to sample the same anatomical areas in two differently stained sections. 10 fields of interest were analysed from each side of the section (10 CST and 10 PCP). For sampling protocols of the CST and the PCP refer to Figures 5.1 and 5.2. Similarly, the majority of fields were exported as an image of the computer screen at magnification of 40X and a resolution of 1024x768 (each fields surface area was 0.047).

6.3.2 Axonal counting

All fields of interest were examined by the imageJ software. Since numbers of axons are much higher than OLs numbers, manual counting of axons is very time consuming. Therefore, an automatic counting protocol was established. With imageJ software, some features of the image can be adjusted to optimise and validate automatic counting (Figure 6.2). We validated the automatic counting protocol by comparing it with manual counting. Twenty fields were selected and manual counting was applied. Initially, intraobserver reproducibility of the manual counting in two

occasions was calculated and demonstrated a Pearson correlation of 0.951 and a p value < 0.001.

Several attempts were carried out to adjust the image threshold to produce the strongest correlation between automatic counting and manual counting. The best significant correlation achieved compared to manual counting was; Pearson correlation r = 0.882, p value < 0.001. The threshold value that was associated with best correlation was recorded in a "plug in". The plug in is a written programme, which can automatically perform the previous steps as shown in Figure 6.2.

6.3.3 Calculating total number of axons in the CST and PCP

The mean density of axons in the 10 fields that were exported from the CST of each subject was multiplied by the CST areas of that subject. Similarly, the total number of axons in the PCP was calculated.

6.3.4 Identifying myelin status of each field

After counting axons, the myelin status of each field was identified using the method mentioned in Section 4.3.7. Then fields were categorised according to the myelin status into NAWM, PMWM, and DMWM and the mean density of axons in each areas was estimated.

6.3.5 Ratio of oligodendrocytes to axons

We have previously applied the sampling protocol of the CST and the PCP on the CA II stained sections. Numbers of OLs obtained from each field were compared with numbers of axons in the corresponding field. The obtained ratio of OLs to axons was calculated in the three differently myelinated areas and a comparison was made between PPMS and SPMS.

6.3.6 Statistics

The field number, myelin status and axonal count were exported to SPSS. The student t-test was used to compare the means. Pearson correlation was used to test correlations. Controlling for the independent factors, partial correlation was used to look for correlation between disease duration and axonal density. Linear regression was also used to examine the effects of independent factors such as age, gender, disease duration, cord level, and disease subtypes, on axons density in the three differently myelinated areas.

6.4 Results

In total, we exported 713 fields from the CST and 721 fields from the PCP. Similar to the CA II stain, the NE14-stained sections do not show areas of altered myelin composition. Therefore, areas of abnormal myelination were identified using the MBP stain (Figure 6.3). The numbers of analysed fields according to disease type and myelin status are summarised in Table 6.1.

Table 6.1: Summary of the examined fields for axonal analysis

Type of the disease	Myelin status of the field	Number of examined fields PCP	Number of examined fields CST
Normal control	Normal	99	105
PPMS	Demyelinated	54	45
	Partially myelinated	42	41
	Normally appearing from MS	208	224
SPMS	Demyelinated	84	69
	Partially myelinated	64	97
	Normally appearing from MS	170	132

6.4.1 Quantification of axons in the CST and the PCP in healthy controls

The surface area of the CST and the PCP varied between different cord levels, which seem to be affected by the amount of axons that enter or leave the tract. The CST surface area is largest in the lower cervical segment and smallest in the upper

lumbar segment. The PCP area is largest in the lower cervical region and smallest at the thoracic segments (Table 6.2).

The mean density of axons in the CST was $3.9x10^4/mm^2$ and was higher than that in the PCP, which was $2.9x10^4/ mm^2$ (p value < .001, irrespective of cord level). Total numbers of axons in the CST and the PCP were highest in the lower cervical cord, and were 0.7 $x10^6$ and $0.4x10^6$ respectively. Controlling for age and gender, the main cord level has a tendency to affect the total number of axons in the CST (p = 0.061) but not the PCP (p = 0.843).

6.4.2 Quantification of axons in MS irrespective of myelin status

The CST surface area in MS was smaller compared to normal controls in various levels. This reduction was significant in the upper cervical, lower cervical and lower lumbar (Table 6.2 and Figure 6.4). In comparison, the area occupied by the PCP did not differ from controls at any cord level.

In the two examined tracts, there was significant reduction in the density of axons, compared to the corresponding area in healthy controls. The mean axons' density in the CST of MS was $2x10^4/ mm^2$ (compared to controls $3.9x10^4/mm^2$, p < 0.001) and in the PCP was $2.4x10^4/ mm^2$ (compared to controls $2.9x10^4/mm^2$, p = 0.003). From the data above, it appears that there is greater reduction of axons in the CST (Figure 6.4).

When different cord segments were examined, it appeared that axonal pathology in the CST affected all levels almost to the same degree. In comparison, axonal loss in the PCP demonstrated less reduction in axonal density, especially at the lower cord segments (Table 6.2).

Since we have reductions in the axonal density and the surface area, the total number of axons also demonstrated significant reduction. The average total number of axons in all spinal cord levels in the CST of controls was $0.3x10^6$ and in MS was $0.1x10^6$ ($p < 0.001$). In the PCP the mean total number was $0.2x10^6$ in controls and $0.2x10^6$ in MS ($p = 0.211$). The total number of axons is best assessed when the segment level is considered. Compared to controls, this reduction was significant in the upper cervical, lower cervical, and lower lumbar segments of the CST, while the total number of axons in the PCP was not different from controls in all segments. Detailed results are mentioned in Table 6.2.

Table 6.2: Comparison between MS and controls

Location of WM	Segment category	Sample type	Density of axons			Surface area (mm^2)		Total number of axons	
			axons/mm^2	Percentage of reduction	P	Value	P	value	P
CST	Upper cervical	Control n = 2	29939	43%	.000	15.36	.005	455947	.021
		MS n =11	17095			8.39		152096	
	Lower cervical	Control n = 2	36721	57%	.000	17.45	.008	760383	<.0001
		MS n = 6	15654			7.17		117786	
	thoracic	Control n = 1	23816	22%	.303	5.42	.758	129081	.846
		MS n = 9	18521			6.11		115454	
	Upper lumbar	Control n = 2	38205	39%	.000	3.40	.527	134962	.541
		MS n = 10	23496			4.25		99093	
	Lower lumbar	Control n = 4	48950	49%	.000	5.47	.001	264256	.015
		MS n = 7	25002			2.50		64947	
PCP	Upper cervical	Control n = 2	22719	27%	.038	14.50	.256	306164	.195
		MS n =11	16684			8.22		134569	
	Lower cervical	Control n = 2	23728	42%	.000	16.40	.254	411491	.074
		MS n = 6	13813			12.72		194884	
	thoracic	Control n = 1	26206	14%	.381	5.48	.159	143611	.494
		MS n = 9	22436			8.77		185415	
	Upper lumbar	Control n = 2	39190	12%	.349	7.74	.339	277042	.486
		MS n = 10	34355			9.81		369080	
	Lower lumbar	Control n = 4	29025	00%	.163	9.01	.133	251609	.706
		MS n = 7	34329			5.85		215588	

6.4.3 Pathology of axons in the DMWM

The mean density of axons in the DMWM of the spinal cord of global MS sample was $1.9X10^4/mm^2$. This was significantly less than normal controls ($p < 0.001$) and NAWM ($p < 0.001$). The two tracts did not show a difference in axonal density of the demyelinated areas. The mean axonal densities in the DMWM of the CST and the

PCP were $2x10^4/mm^2$ and $1.9X^4/mm^2$ respectively (p value 0.642). Density of axons in the DMWM of the two tracts showed significant reduction compared to the corresponding area of controls ($p < 0.001$ for the two comparisons)

By categorising results according to the disease subtype, there was no difference between PPMS and SPMS in the average axonal density in the DMWM of the two tracts (SPMS $1.8x10^4$, PPMS $2.1x10^4$, $p = 0.234$). In PPMS there was 38% reduction in the axonal density compared to normal controls and 22% compared to the nearby NAWM. In SPMS there was 47% reduction compared to normal controls and 14% compared to NAWM (Figures 6.5 and 5.6)

6.4.4 Axonal density in the PMWM

The average density of axons in the PMWM of the two tracts was $1.6x10^4$ (compared to controls $3.4x10^4$, $p < 0.001$). The PMWM has axonal density of $1.2x10^4$ in the CST and $2.1x10^4$ in the PCP (compared to controls, p value was < 0.001 for the two tracts). Details are mentioned in Table 6.3 and Figures 6.5 and 6.6.

Compared to controls, there was reduction in axonal density in the PMWM of SPMS in the CST ($p <0.001$) and the PCP ($p =0.001$). Similarly, in PPMS, the PMWM has reduced axonal density compared to controls in the CST ($p < 0.001$) and the PCP ($p = 0.015$).

6.4.5 Axonal density in the NAWM in MS

The NAWM of MS showed significant reduction in axonal density compared to controls. Regardless of the type of the tract, the average density of axons in control spinal cords was $3.4x10^4$ and in NAWM of MS was $2.4x10^4$ ($p < 0.001$). When the two tracts were compared to each other, there was significant difference in axons'

density in the NAWM of CST ($2.2x10^4$) compared to the PCP ($2.6x10^4$) ($p = 0.002$). When the densities of axons in NAWM of the two tracts were compared with controls separately, the CST showed significant reduction compared to controls (CST axonal density in controls was $3.9x10^4$, $p < 0.001$), while there was no difference between the NAWM of PCP in MS and the PCP of controls (PCP axonal density in controls was $2.9x10^4$, $p = 0.141$). We calculated previously that axonal density in the PCP of MS was reduced compared to controls when the DMWM and the PMWM were included in the sample. This implies that reduction of axons in the PCP is due to demyelinating lesions, whereas axons of the NAWM seem to be preserved. In the CST reduction affected the DMWM and the NAWM (Figures 6.5 and 6.6).

Table 6.3: Comparison between PPMS and SPMS in axonal density in the PMWM and DMWM in the cervical cord.

Myelin status of the field	Location of WM	Type of the disease	Axonal density in the field	P value
Demyelinated	CST	PPMS	$2.0x10^4$	0.868
		SPMS	$2.0x10^4$	
	PCP	PPMS	$2.2x10^4$	0.050
		SPMS	$1.7x10^4$	
Partially myelinated	CST	PPMS	$1.9x10^4$	0.017
		SPMS	$0.8x10^4$	
	PCP	PPMS	$2.2x10^4$	0.574
		SPMS	$2.0x10^4$	
Normally appearing from MS	CST	PPMS	$2.6x19^4$	<0.001
		SPMS	$1.6x10^4$	
	PCP	PPMS	$2.7x10^4$	0.156
		SPMS	$2.4x10^4$	

Regardless of the cord level and type of the tract, the mean axonal density in the NAWM of SPMS was $2.1x10^4$ and in PPMS was $2.7x10^4$ ($p < 0.001$). In both disease subtypes, axonal density was reduced compared to controls ($p < 0.001$ for the two comparisons). When the two tracts are examined separately, the density in the NAWM of CST of PPMS was $2.6x10^4$ and in SPMS was $1.6x10^4$ ($p < .001$). The mean axonal density in the NAWM of PCP of PPMS is $2.7x10^4$ and in SPMS is $2.5x10^4$ ($P = 0.16$). It is important to note that there was no apparent axonal loss in the NAWM of ascending tracts compared to descending tract (Table 6.3).

6.4.6 Ratio of oligodendrocytes to axons in cross sections

As we have already demonstrated, axonal loss includes the lesion centre, the lesion border of the two tracts and the surrounding NAWM of the CST. The OLs: axons ratio may give an idea of the degree of loss of each element in the lesion and around the lesion.

In controls, the ratio of OLs to axons was 0.6% (in the CST was 0.72% and in the PCP was 0.41%). In MS, the ratio varies according to myelin status of the fields. In the NAWM, the ratio was 0.6% (in the CST was 0.6% and in the PCP was 0.56%). Results of OLs to axons ratio obtained from the NAWM of the CST and the PCP were similar to those from normal controls. This may indicate that in NAWM, axons and OLs are affected to the same degree (Table 6.4).

However, in the PMWM and the DMWM, OLs' loss seems to be more extensive relative to axonal damage. In the PMWM, the ratio was 0.39% (0.48% in the CST and 0.29% in the PCP). In the DMWM, the ratio was 0.06% (0.1% in the CST and 0.02% in the PCP). By comparing the two disease subtypes, there was no significant difference between OLs to axons ratio in the NAWM and the PMWM. However, the comparison was significant in the DMWM where SPMS has considerably higher OLs: axons ratio (Table 6.4).

Table 6.4: ratio of OLs to axons in SPMS and PPMS

Myelin status of the field	Type of the disease	OLs:Axons %	P value
Demyelinated	PPMS	.0252	.012
	SPMS	.0740	
Partially myelinated	PPMS	.4660	.161
	SPMS	.3484	
Normally appearing from MS	PPMS	.5721	.660
	SPMS	.6103	

6.4.7 Effect of independent factors

Effects of independent factors, such as disease duration, gender, type of disease, and myelin status, on axonal density was assessed by linear regression. There was

significant effect of gender (males were more affected, p = 0.002), type of the disease (p <0.001), and myelin status (p < 0.001) on axons' density. There was no significant effect of disease duration on the global sample, controlling for other factors (p = 0.166).

However, when correlation between disease duration and axonal density was compared in each differently myelinated area, results varied according to the area examined. Partial correlation controlling for age, gender, disease subtype, type of the examined tract, and cord level, was used to study effects of disease duration on axons. Controlling for the previous factors, the correlation was only significant in the NAWM (correlation = -0.217, p = <.001). There was no relation between disease duration and axonal loss in the areas of complete demyelination (correlation = 0.002, p = 0.969) and areas of partial myelination (correlation = 0.72, p = 0.267). This may implicate that axonal injury within the lesion is an early process, while axonal loss in normal areas is more progressive.

Correlation of disease duration with total number of axons was calculated in each tract controlling for age, gender, disease subtype, and cord level. Results showed significant negative correlation with the CST (correlation = -0.0437, p = 0.006) and with the PCP (correlation = -0.487, p = 0.002).

6.5 Discussion

The present study quantified axons in controls and progressive MS. We have noticed variation in the CSA of the CST and the PCP according to the level of the cord. A significant number of descending axons leave the CST at the cervical cord to supply the upper limbs. This results in reduction of the CSA of the CST caudally. With

regard to the PCP, a significant number of ascending axons from the upper limbs enter the cervical spinal cord resulting in increase in CSA of the PCP rostrally.

There was also significant effect of cord level on the total number of axons in the CST, but not the PCP. It is important to mention that considerable number of axons in the CST run long distance in the tract and leave at certain segments. The majority of axons of the PCP leave the tract after a few segments. Therefore, axons continue entering the PCP from peripheral nerves and leaving the PCP to synapse in the nucleus proprius or Clark's nucleus. This may explain the insignificant effect of cord level on the total number of PCP axons. In normal controls, density of axons was greater in the CST compared to PCP, which is probably due to smaller axons in the CST compared to the PCP [295].

In MS, initially we estimated the CSA of the tracts and the average density of axons irrespective of myelination status. There was significant reduction in the CST area compared to controls in more than one level of the CST. In comparison, in spite of significant demyelination affecting the PCP, there was no significant reduction in its CSA at any of the examined cord levels. A previous study has demonstrated similar results; Deluca et al 2004 examined 55 cases of progressive MS and 32 controls and found that atrophy of the CST was more extensive than PCP [122]. This may be explained by the greater reduction of axons in the CST calculated in this study. The significant contribution of chronic axonal loss in causing spinal cord atrophy has been confirmed previously in EAE [341]. Both tracts have axonal loss compared to the corresponding area of controls, but we found that the CST appeared to have greater reduction of axons compared to the reduction that affected the PCP. In addition, axonal loss in the CST was observed in all segments of the cord, which can be the

leading cause of atrophy that affected the CST in multiple segments. These findings may explain the predominance of motor symptoms over sensory symptoms in MS.

6.5.1 Axonal loss in the demyelinated lesions

Axonal density was reduced in the DMWM compared to controls and the NAWM in the two tracts. There was no difference between axonal densities in the DMWM between the two tracts, suggesting similar local destructive process. There was 43% reduction in axonal density in the DMWM compared to normal controls. It is possible that the main bulk of this axonal loss in DMWM of chronic lesions occurred early during the evolution of the plaque. This is because MRI [243] and histopathology [69, 324] studies showed significant axonal loss in early MS and acute MS. In addition, the correlation between axons' density in the DMWM and disease duration was insignificant in this study, suggesting that local loss of axons in MS lesions is independent of chronological progression of the disease and may depend mainly on acute destructive event. In the early stages of MS, local axonal damage appeared to correlate with inflammation. Acute axonal loss was found to be significant in early MS lesions by tracing APP [324, 326]. On the other hand, acute loss of axons within chronic plaques was found to be limited compared to acute plaques [324, 326].

So far, the longstanding effect of acute axonal loss or dysfunction within MS lesions is unknown. A 6-year longitudinal MRI study on a single patient with RRMS showed significant correlation between changes in NAA expression and a major relapse that was followed by recovery [319]. Therefore, changes in NAA reflect axonal function rather than axonal loss. This is because there is frequently complete clinical recovery after the attack, and there is also histopathological recovery of the lesion by remyelination, ie fate of acutely damaged axons is unclear.

6.5.2 Axonal density in the PMWM

When we moved from the lesion centre, data from the lesion border showed variable results. A previous study also showed variable axonal densities at the MS lesion border [324]. We had also variable results from counting OLs in the partially demyelinated areas and the lesion border. It is important to acknowledge that the included partially myelinated areas in this study depend mainly on the MBP stained section viewed under light microscopy, therefore the nature of this partial demyelination is unknown. These areas may represent partially myelinated areas, partially remyelinated areas or areas of Wallerian degeneration. Although most lesions in our study have been confirmed previously to be chronic [124], the level of lesion activity at the border may vary even in chronic lesions. Therefore, variability of results at the lesion border and the partially myelinated areas may be due to different degrees of inflammation or activity.

6.5.3 Axonal density in the NAWM

The present study illustrated further that loss of axons is not only restricted to the injured area but extends to affect NAWM. Axonal loss continues to be an important feature of progressive MS in the NAWM [242]. A post-mortem human histopathology study also demonstrated axon loss in the NAWM of the cervical segment [342]. It is believed that axonal loss in the NAWM is quite an early process. Bjartmar and colleagues has observed axonal loss in the NAWM of a patient who died within 8 months from disease onset (case study) [69]. They estimated 22% reduction in axonal density in the NAWM of the descending tract of the MS spinal cord. In comparison, the present study showed 42% reduction in axonal density of the NAWM of the descending tract in the cervical level. These data imply that axonal loss in the NAWM is an early and progressive process. Interestingly, we calculated significant correlation between axonal loss in NAWM and disease duration. This may explain

the rise in axonal loss from 22% (in the NAWM of a patient with disease duration of 8 months) to 42% (in the NAWM of progressive MS with mean disease duration of 24 years in this study). Bjartmar and his colleagues did not find reduction in axonal density in the ascending tract of the cervical spinal cord in that case report of acute MS. In this study there was 29% reduction in axonal density of the NAWM of the PCP at the cervical segment (there was no difference when all levels are included).

Axonal loss of the NAWM can be due to effects of Wallerian degeneration. There are two pieces of evidence to suggest this hypothesis: firstly, there is greater loss of axons in the NAWM of descending tracts compared to ascending tracts. This has been reported previously in chronic MS [122] and acute MS [69]. Secondly, an interesting finding in Bjartmar case study of acute MS is that there were myelin sheaths in the NAWM with no axons inside [69], which is the histopathological picture of Wallerian degeneration. Likewise, the NAWM areas in the present study are areas with apparently normal myelin sheaths, but we do not have evidence that any axons are contained in these myelin sheaths.

Another assumption is that MS is primarily an axonal disease and that demyelination does not have great effect on axonal survival [326]. It has previously been demonstrated that axonal loss in the NAWM of MS affects mainly the small diameter axons. Three studies suggested selective loss of small axons in MS in the CST [122, 318, 342] and one in the PCP [122]. Studies on healthy controls reported that density of axons is normally reduced with age progression. In addition, axonal loss that occurs as part of the normal degenerative aging process was found also to be selective for small myelinated axons [343]. In the present study, comparison between the NAWM of MS and the normal WM from healthy subjects was controlled for age, gender, disease duration, and cord level. This means that the presumed

loss of small axons is more prominent in MS compared to the age- matched healthy controls. However, it is not certain if axonal loss in NAWM of MS is only acceleration of normal aging degenerative process or due to a mechanism that is peculiar to MS. Conversely, another hypothesis says that there is no selective axonal loss in MS but the surviving axons undergo swelling [124].

6.5.4 Variation between the two tracts

The previous data suggests that demyelination results in similar local pathology within the lesion itself between the two tracts, but the following effects of neuronal degeneration in the NAWM may vary between the ascending sensory and the descending motor tracts.

Loss of axons in the NAWM of CST affected all levels from the cervical cord to the lumbar cord in spite of less demyelination in the lower cord regions. In contrast, the ascending PCP was only affected in the upper cord area. This is in agreement with Deluca et al (2004) findings. It was shown that reduction of axonal density in MS affects the CST in all levels but affects only the upper levels of the PCP. There are three possible reasons that may explain these findings:

Variation in the tracts' anatomy

A significant number of axons in the CST of the lower segments run through the whole length of the spinal cord. Long axons within the CST are vulnerable to demyelinating lesions along their course. This can be supported by the fact that lower limbs are more affected by paraplegia than upper limbs.

In comparison, axons of the PCP start peripherally and enter the spinal cord dorsally. Most axons of the PCP ascend a few segments then leave the tract to synapse in the

Clark's nucleus. If a demyelinating lesion affects the upper cervical segment, this will likely affect the upper limb sensation, which is a notable finding in MS and has been discussed before [122]. This is because a significant number of PCP axons that come from the lower limbs already gave sensory information to the Clark's nucleus.

Variation in type and timing of neuronal degeneration between sensory and motor tracts

In the introduction, I discussed the normal response of the human CNS to injury, and emphasised that Wallerian degeneration can be faster than retrograde degeneration. In addition, timing of retrograde degeneration depends on the proximity of the lesion to the cell body, being slow in lesions far from the neuronal cell body [344]. Consequently, the abundant cervical lesions will cause Wallerian degeneration of distal part of the CST axons in the thoracic or lumbar cord. Alternatively, similar lesions in the upper cervical segment in the PCP will presumably cause Wallerian degeneration proximally in the brain, and much slower retrograde degeneration distally in the lower cord. Because of the rarity of lesions in the caudal segments of the cord, Wallerian degeneration of the NAWM of ascending tracts will be limited. One can expect that, in response to a lesion in the cervical cord, pseudounipolar cells in the dorsal root ganglion of the cervical cord will degenerate faster than those in the lumbar region. Such degeneration may cause axonal loss in the nearer NAWM and may explain our finding that there was 29% loss of axons in the NAWM of the PCP in the cervical cord only and not in the rest of the cord.

In order to test this assumption, I conducted a quick comparison in the NAWM between the cervical and the lumbar cord in each individual. Individuals with a lower density of axons in the NAWM of CST in the cervical cord tend to have lower density

in the lumbar cord. This was not the case in the PCP, which showed apparent disagreement (Figure 6.7).

Different axonal sizes in the two tracts

The presumed selective axonal loss in MS, by causing damage to small axons less than 3.3 μm [342], may result in variation between the two tracts. This is because the PCP has mainly large heavily myelinated fibres of 10 μm and more [345, 346]. Most axons of the PCP are large diameter axons of the A alpha A beta fibres [347]. In comparison, 90% of CST axons are 1-4 μm in diameter [295, 348] making the CST axons susceptible to the hypothesised selective axonal loss.

6.5.5 Variation between the two disease subtypes

In addition to the differences between PPMS and SPMS in degree of atrophy, demyelination, and OLs' pathology, there seem to be differences in the amount of axonal loss. SPMS demonstrated less axonal density within the NAWM and the PMWM, while there was no difference in the DMWM. The difference between the two disease forms was more prominent in the CST. A study carried out in 2000 revealed significant loss of axons in acute MS lesions in SPMS but not in PPMS. Lovas et al reported 60% reduction in density of axons in SPMS, where small axons are lost more than large axons [342].

Tallantyre et al compared axonal loss in the DMWM with the NAWM in the cervical cord, and found that PPMS shows greater loss of axons compared to SPMS. In this study, we found that PPMS shows greater loss of axons in the DMWM when compared with the surrounding NAWM (PPMS 22% loss, SPMS 14% loss). When compared with normal controls, however, SPMS shows greater loss of axons (PPMS

38% loss, SPMS 47% loss). This can be explained by the finding that SPMS has significantly greater loss of axons in the NAWM.

It is possible that the difference between the two diseases in axonal density is due to difference in inflammation and disease activity. The preceding relapsing remitting phase of SPMS, which can extend up to 20 years, may result in greater inflammation-induced axonal damage. There is some evidence to support this assumption; acute axonal damage correlated significantly with inflammation activity, SPMS showed greater degree of acute axonal loss, even in active lesions [72], and early axonal loss was observed in the NAWM of acute MS [324].

The progressive forms of the disease are also different in the degree of demyelination in the brain, which may affect survival of axons. Greater demyelination of the brains and the spinal cords in SPMS may result in a greater degree of degeneration caudally. In support to this hypothesis, we found that the difference between the two disease forms in axon density in the NAWM was only significant in the descending tract and not the ascending tract, ie axons that traverse MS lesions in the brain or cervical segment continue to be part of NAWM in lower cord levels. Since we have more areas of demyelination within the brains and spinal cords in SPMS, the caudal regions of SPMS can be affected more extensively than same areas in the PPMS, in particular the NAWM.

Patients with MS have variable clinical course according to disease subtype. However, the EDSS of all subjects in this sample has been identified and was found to be similar [142]. Although axonal loss correlated with disability, it is possible that greater difference in axonal density is required between the two subtypes before a significant difference in disability can be observed. In EAE, up to 30% of axons can

be lost before irreversible motor deficits appear [349]. NAA expression in the brains of MS patients was found to be significantly reduced early in the disease, and even before the appearance of clinical symptoms [243]. A previous study reported 64% reduction in axonal density of MS lesions without evidence of clinical disability [305].

Degree of axonal loss in any tract is not the only factor that may affect disability. The extent of fibres' decussation in CNS tracts is believed to be crucial in lessening the clinical manifestations of lesions in the CNS. In rare cases there is complete decussation, while in others, there is no anterior CST [339]. Therefore, the same lesion may produce a variable degree of disability in different subjects depending on the degree of pyramidal decussation (Figure 6.1) [340]. For a randomly selected sample (irrespective of degree of decussation as in our sample), greater difference in axonal loss may be needed to produce a noticeable difference in disability between two groups, ie the calculated difference in axonal loss between PPMS and SPMS in our study may not be enough to produce a recordable difference in disability.

The ratio of OLs to axons was calculated as the percentage of OL number to axon number in each field. It is important to state that this ratio does not represent the actual ratio of OLs to axons. One axon can be myelinated by too many OLs along its course. Ratio calculated in this study reflects number of OLs and axons in cross sections and not in the three dimensional space. However, the calculated ratio may reflect the degree of OLs compared to axonal loss. The ratio suggests that axonal loss and OL loss happen equally in the NAWM, while in the demyelinated areas, there is significantly less destruction of axons compared to OLs.

In this study selection of fields was governed by unbiased sampling process, regardless of the myelin status, as the NE14 stain does not show areas of

demyelination clearly. The automatic counting of axons added more to the objectivity of the study. However, automatic counting may be affected by the obliquity of the section

To validate our results and to exclude effects of shrinkage and atrophy in MS cases, we have calculated the total number of axons in the CST and the PCP. Total number of axons may reflect degree of disability better than density of axons. For example, the total number of axons on one side of the CST in the upper cervical cord of controls was 0.7 million and in MS was 0.1 million (85% reduction). In comparison, the mean axonal density in the CST in the lower cervical cord of controls was ≈ 0.4 million and in MS was ≈ 0.2 Million (57% reduction). Total number of axons in MS was calculated by multiplying the mean density of axons with the CSA of the tract. It is therefore affected by the calculated CSA. It is possible that degree of atrophy in the CST is due to loss of other elements, such as glial cells and neuropil, and not only due to axons.

6.6 Conclusion

This study has shown variation in the amount of axonal loss between ascending and descending tracts in the NAWM of MS spinal cords. There was no difference between the two tracts in the DMWM. This may indicate the there is difference in the extent and timing of neuronal degeneration between ascending and descending tracts. This may imply that axonal survival in the NAWM is dependent mainly on degeneration and not due to local effects of the plaque.

In spite of the significant demyelination of the PCP, there was no significant atrophy of the tract compared to the CST. This may represent evidence that atrophy is largely

affected by loss of axons in the NAWM, especially because loss of axons in the NAWM of the PCP was found to be limited.

We also demonstrated that SPMS has a greater degree of axonal loss in the NAWM. This may be related to the observation in the previous chapter, when we found that there was also greater loss of OLs in the NAWM. Although there is an agreement between axonal loss and OLs in the NAWM (OLs: axons ratio in MS does not differ from controls when there was significant loss of both elements), it is not well known which one preceded the other. Possibly examination of the NAWM in acute MS for OLs' loss may answer this question.

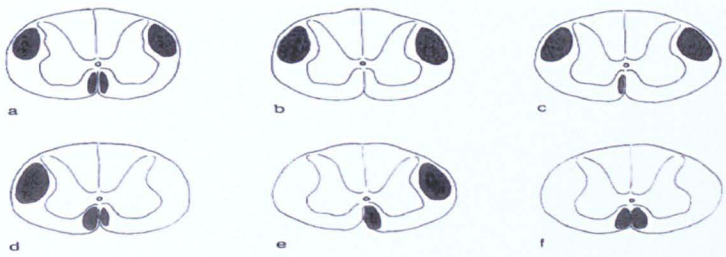


Figure 6.1: Normal anatomical variation in degree of the CST decussation [340].

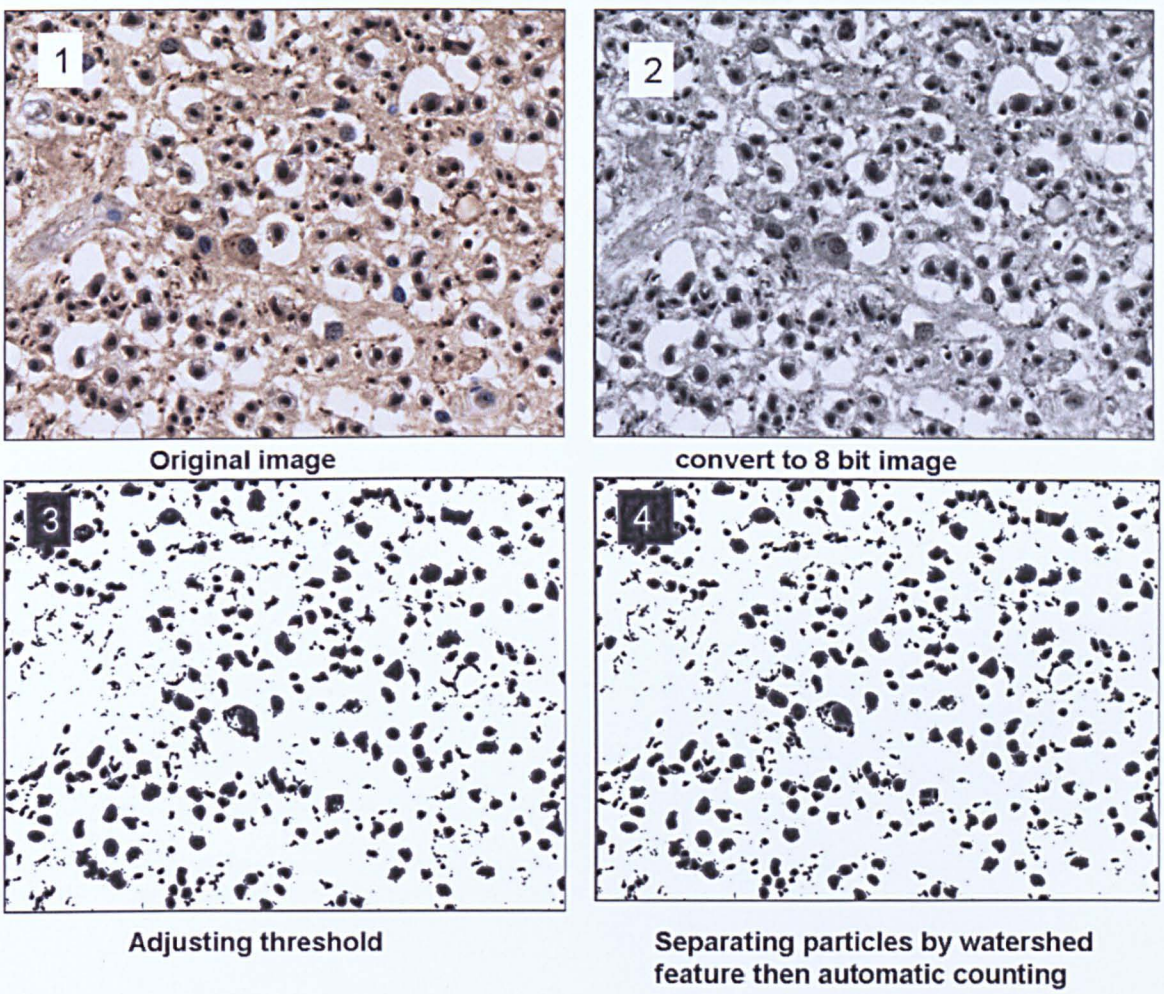


Figure 6.2: Stages of automatic counting: the original image (1); coloured images are then converted to grey images (8 bit images) (2); Threshold of the image is then adjusted (3). Separation of particle is done automatically by the software (4). All particles in image 4 are then counted automatically.

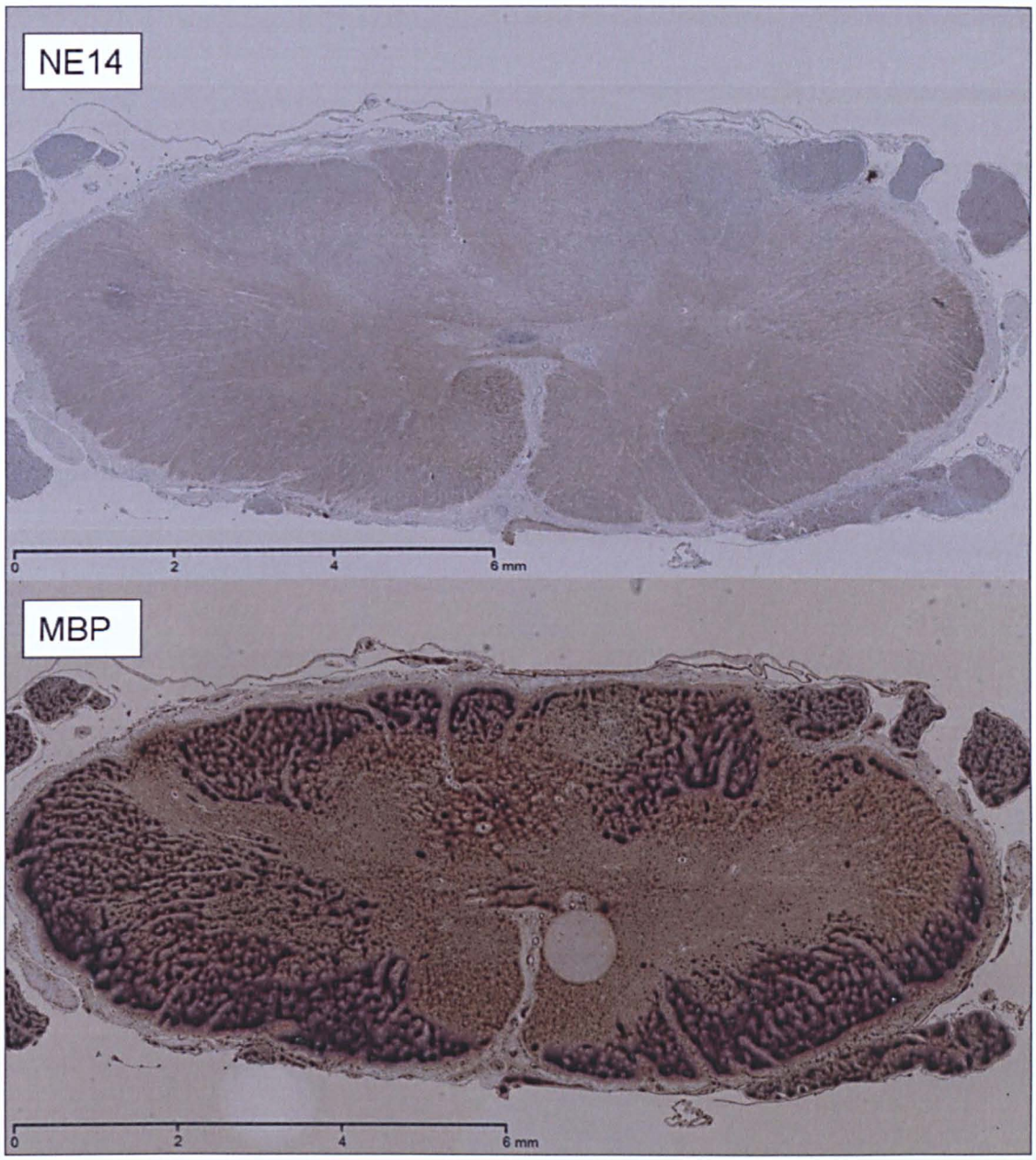


Figure 6.3: Human spinal cord cross section. Images were taken from the same subject and same level. Note that demyelinated regions are not easily identified on NE14 stained sections

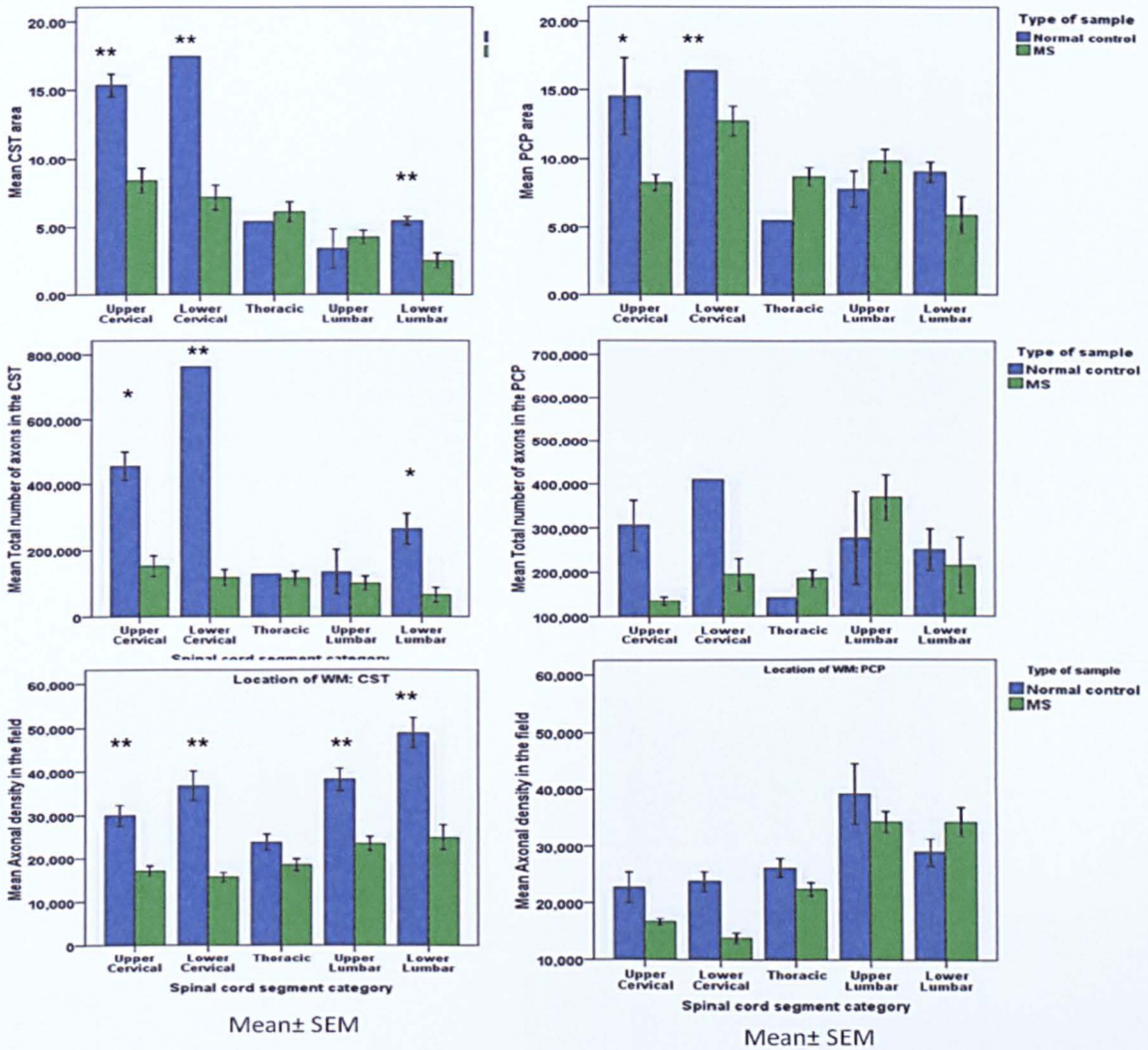


Figure 6.4: Bar charts demonstrate the variations between MS and controls in the two tracts. The bars represent the mean CSA area, the mean total number of axons and the mean axonal density ± SEM. The p values < 0.05 are indicated by one asterisk and the p values <0.01 are indicated by two asterisks.

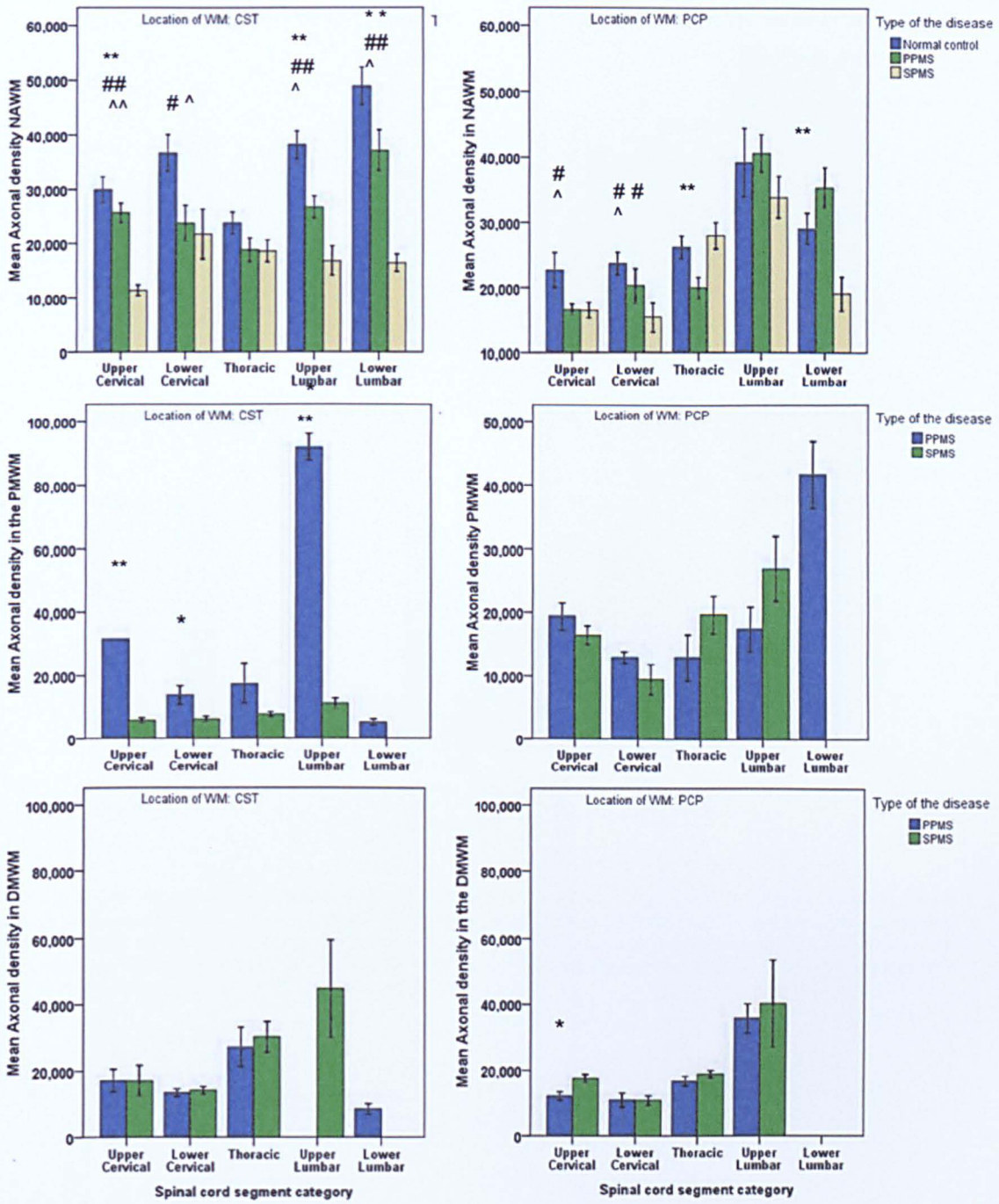


Figure 6.5: Bar charts showing the differences between PPMS and SPMS in axonal density in NAWM, PMWM, and DMWM. The bars represent the mean axonal density in the NAWM, PMWM, and DMWM ± SEM. The significant difference between SPMS and PPMS is indicated by asterisks *, the significant difference between controls and SPMS are indicated by the number sign #, while the difference between controls and PPMS is indicated by ^. For each of the comparisons the significant differences of p value < 0.05 are indicated by one sign, while the p values < 0.01 are indicated by two signs.

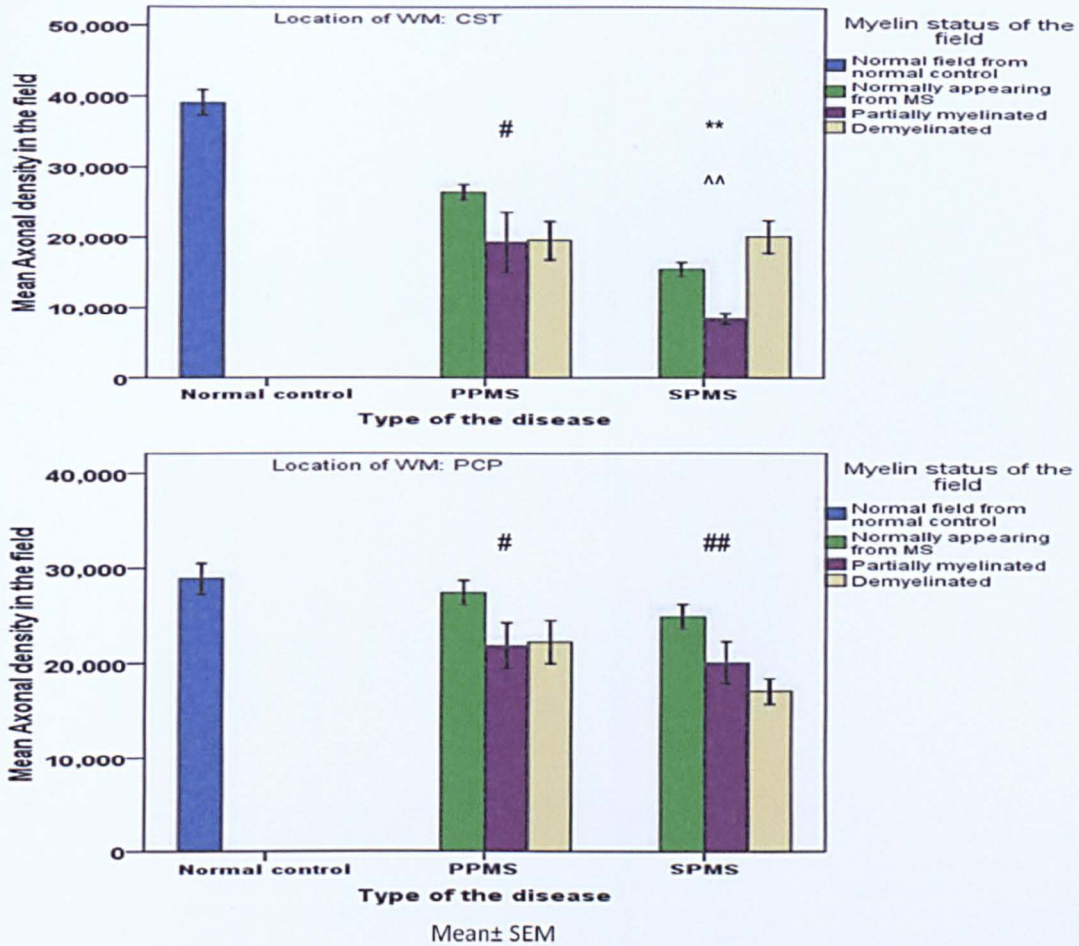


Figure 6.6: Comparison between PPMS and SPMS in three differently myelinated areas regardless of the cord level. The bars represent the mean axonal density in the NAWM, PMWM, and DMWM ± SEM. The significant difference between NAWM and PMWM is indicated by asterisks *, between NAWM and DMWM is indicated by the number sign #, and between PMWM and DMWM is indicated by ^. For each of the comparisons, the p values < 0.05 are indicated by one sign, while the p values < 0.01 are indicated by two signs

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Chapter 7: Pathology of neurons in the spinal cords of PPMS and SPMS

7.1 Introduction

Neurons are the fundamental units of the CNS, and their preservation is essential to maintain function in view of their inability to regenerate in most CNS locations. MS lesions can affect various GM regions of the CNS, including the cerebral cortex, thalamus, basal ganglia, hippocampus, and the spinal cord GM [106, 350, 351]. MS lesions in the GM lead to loss of myelin, which may in turn induce neuronal loss, neuronal shrinkage [106], and dendritic damage [311]. Neuronal pathology and the subsequent loss of function are possibly responsible for some of the disability in MS. In the brain, cerebral cortex pathology is believed to be responsible for decline in the cognitive functions in MS patients [352, 353].

Researchers usually assess neuronal loss by directly visualizing neurons using histopathologic studies or neuronal culture studies (in vitro), or indirectly by measuring degree of NAA expression by means of MRS (in vivo) [131]. Various types of study have reported neuronal loss that is related to MS, including MRS studies [354-357] and histopathological studies [95, 351, 358]. Additionally neuronal culture studies [354, 359] and experimental models of MS [360, 361] have shown neuronal loss that is related to MS pathology. Neuronal loss can be an early process as shown by the concentration of NAA, which was reduced in the NAGM of the cortex in short duration RRMS [357].

With respect to neuronal pathology, the spinal cord has again received less attention compared to neuronal pathology in the brain. Only one study examined quantitatively neuronal pathology in the spinal cord. The study was carried out in our lab using a

relatively big sample size, but there was no differentiation between PPMS and SPMS in neuronal counts [106]. In this study we have the opportunity to examine the two subtypes of progressive MS in a very well clinically characterised sample.

7.1.1 Causes of neuronal loss

Demyelination

A number of studies have found correlation between demyelination and loss of neurons. The most recent spinal cord study was conducted by Gilmore et al 2009. The study quantified numbers and sizes of motoneurons and interneurons in 38 cases of MS and 22 controls. Neuronal loss was mainly associated with demyelination of the GM. Atrophy of interneurons was also found to be significant and affected all cord segments in both demyelinated and normal appearing GM [106].

A recent study was carried out in 2009 by Papadopoulos and colleagues. Papadopoulos et al examined quantitatively hippocampus autopsy material from 45 cases of progressive MS (mean disease duration was 27.8 years) and 7 controls. The study reported 30% demyelination and 23% atrophy of the archaeocortex (3 layers cortex), which is the area responsible for memory. There was 27-29% reduction in number of neurons compared to controls. In addition, size of surviving neurons was reduced by 17%. Interestingly there was significant correlation between loss of neurons and degree of atrophy [362].

Similar to Papadopoulos study, Wegner et al studied quantitatively autopsy material from the neocortex (6 layers cortex) in 22 cases of MS and 17 controls [358]. There was 10% decline in the thickness of MS neocortex compared to controls. This was associated with 10% reduction in the neuronal number and 47% reduction in the

synaptophysin expression (a marker of synapses). Loss of neurons was associated with demyelinated areas while the normal-appearing regions of the neocortex were not associated with significant pathology of neurons.

Another study has also quantified demyelination, neuronal and synaptic losses in MS cerebral cortex. There was 33% neuronal loss in the cerebral cortex lesions compared to the adjacent normally appearing cortex [351].

Degeneration

Neuronal cell bodies may undergo degenerative changes when their axons are severed in the process of retrograde degeneration. This can affect demyelinated areas and normally appearing areas. Anterograde degeneration (Wallerian degeneration) may result in degeneration of the postsynaptic neuron. It is believed that neurons that do not receive action potentials may degenerate due to de-afferentation [76]. Both retrograde and anterograde degeneration may continue to affect more than one order neuron. Retrograde degeneration has been directly demonstrated in different human CNS regions [97, 363], such as the cochlear system [363], the olivocerebellar pathway [364], and the CST [365]

The effect of retrograde and transynaptic degeneration on neuronal survival in MS has been suggested in a number of studies. A correlation between loss of axons and neuronal changes within the same pathway has been reported by Evangelou et al 2001 [95]. Evangelou quantified numbers and sizes of axons in the anterior optic pathway and compared it with degree of neuronal shrinkage and loss within the geniculate nucleus in 8 MS patients and 8 controls. There was strong correlation between loss of small diameter axons and shrinkage of small neurons. This further

confirms the effect of distant axonal loss on the GM and the subsequent neuronal loss.

Cifelli et al demonstrated atrophy and loss of neurons in the thalamus in both MRS and histopathologic post-mortem analysis (two different samples). There was 30% reduction in the effective neuronal density measured by MRS study compared to 22% reduction in the neuronal density measured by histopathology study [131].

Peterson et al investigated pathology of MS in the cerebral cortex including axonal, neuronal and inflammatory changes. Peterson and his colleagues quantified transected neurites and apoptotic neurons in the cerebral cortex of MS autopsy material. Neuronal apoptosis was found to be significant not only in the acute active lesions, but also in the chronic inactive lesions. Such findings suggest that neuronal apoptosis continues in chronic lesions in the absence of acute immune attack [311].

Moreover, Peterson and colleagues demonstrated changes in microglia morphology. Microglia were seen abutting neuronal cell bodies and possibly causing de-afferentation of these neurons. This may provide initial protection to degenerating neurons from further injury. This is because excitatory impulses can cause further damage to the already injured neuron. A negative effect of glutamate on neuronal survival has been reported previously [77]. But with prolonged de-afferentation, these neurons may degenerate and even be phagocytosed by the microglia.

Other causes

A number of potential causes have been suggested to contribute in neuronal damage in MS. These may include glutamate excitotoxicity, nitric oxide neurotoxicity, or chemokines/cytokines cytotoxicity.

Vericello in 2007 studied effects of excitotoxicity on survival of neurons in the cerebral cortex of MS tissue. Vericello and colleagues evaluated excitatory amino acid transporter expression in lesions of cerebral cortex of 10 autopsy material. The examined protein prevents excitotoxicity by reducing level of extracellular glutamate (increases glutamate uptake). Vericello reported a correlation between activated microglia and loss of excitatory amino acid transporter expression. Activated microglia also correlated with an increase in c-Jun N-terminal kinase expression (a marker of neuronal excitotoxicity). The main conclusion from the study was the possible significant role of excitotoxicity in causing neuronal and synaptic losses.

Diffusion of harmful proteins from the plaque to the CSF may induce distant neuronal loss. Within MS plaques, macrophages produce proteases which cause tissue destruction. These soluble factors may include antibodies against neurons. In fact, a number of antibodies against neurons were reported in the sera of MS patients [366-368]. In addition to antibodies, soluble cytokines and chemokines may induce neuronal injury in cultures such as TNF-alpha [369-371].

This has been reported in another study in 1998 by Alcazar et al. Neuronal culture was treated with CSF from three groups of patients; worsening PPMS, stable PPMS and non-inflammatory neurological diseases. Apoptosis was only demonstrated in cultures treated with CSF from worsening PPMS [372].

A more recent study by Cid et al (2002) investigated the relation between MS relapses and the degree of apoptosis of cultured neurons. A sample of 24 patients with relapsing disease was studied. The CSF was taken during relapses and added to the cultured neurons. Increased apoptosis was induced by the CSF that had been

taken during the relapses that were associated with poor recovery from the attack. In addition the CSF, which was taken from relapses that were associated with MRI findings, induced the greatest degree of neuronal apoptosis [354].

Xiao et al (1996) compared CSF effects on inducing nitric oxide release from glial cultures between patients with MS and patients with other CNS diseases. 34% of MS cases stimulated glial cell culture to produce nitric oxide compared to less than 10% from other diseases [373]. In relation to nitric oxide neurotoxicity, three mechanisms have been postulated; direct toxicity, peroxynitrate formation from superoxide anion, and via elevation of intracellular cGMP and induction of programmed cell death [374].

7.2 Aims and Hypothesis

Neuronal loss has been reported in a number of studies, but most of these studies were conducted at the level of the brain GM. Considering differences between the two progressive subtypes in demyelination, atrophy, OLs' quantification and axonal loss, this study will investigate if there is any difference between PPMS and SPMS in neuronal pathology. Neuronal survival may be affected by inflammation, demyelination and OLs' loss, which occur in PPMS and SPMS in varying degree.

This has been achieved by examining human spinal cord sample. Numbers and sizes of neurons were estimated in the VH of DMGM and NAGM. Effect of demyelination on neuronal number and sizes were studied. Comparison was made between PPMS and SPMS in various levels of the cord.

7.3 Material and methods

Human autopsy material was analyzed including MS and controls. MS tissue includes material from one source which is the MS Society tissue bank (PPMS n =13,

SPMS n = 14), while controls were collected from three sources; MS Society tissue bank (n = 5), Oxford Radcliffe (n = 6), and Netherlands brain bank (n = 2). Summary of the included sample is in Table 7.1. For this study, we used the NE14-stained slides to quantify neurons. These slides have been used previously to quantify axons within the WM. As we have mentioned, we have examined one slide of 5 μm thickness from each paraffin-embedded block. The NE14 stain traces the neurofilaments in the axons and neuronal cell body. The myelin status of the VHs has been assessed using MBP stain in the previous chapter.

Table 7.1: Summary of the sample used for quantification neurons

Type of the disease	Gender		Age (years	Duration of the disease (years)	Post-mortem delay (hours)
Normal control	male	Minimum	33		22
		Maximum	88		26
		Mean	60		24
	female	Minimum	41		9
		Maximum	93		33
		Mean	67		17
PPMS	male	Minimum	54	16	5
		Maximum	92	54	20
		Mean	74	35	10
	female	Minimum	45	5	6
		Maximum	77	37	87
		Mean	61	20	24
SPMS	male	Minimum	40	9	8
		Maximum	73	47	23
		Mean	57	25	14
	female	Minimum	39	15	7
		Maximum	78	50	27
		Mean	57	23	14

7.3.1 Quantification of neurons

The digital slides of the NE14 stain were used to perform quantification measurements. On each slide and at low power, the surface area of the VHs was estimated. The VH area includes the area anterior to a horizontal line from the anterior border of the GM commissure [106]. The dorsal part of the VH between the GM commissure and the central canal has been excluded because it contains numerous small neurons that can be difficult to differentiate from glia [106].

At magnification of 40X, the area was scanned manually for the presence of neurons. The considered neurons were identified by having a large cell body, Nissl bodies, and presence of nucleolus. Only neurons with visible nucleolus were considered in the analysis. This is because the nucleolus is the smallest visible part of the neuron.

For each neuron, the boundary was identified manually and the software automatically calculates the CSA of the neuron. The axon and the dendrites were not included in calculation of the neuron CSA. The maximum and the minimum diameters of the neuron were measured. The minimum and the maximum diameters of the neurons pass through the nucleolus.

7.3.2 Classification of neurons

According to the maximum and the minimum diameters, neurons were classified into motoneurons and interneurons. Similar to the previously used criteria [106, 375], motoneurons include all neurons with the maximum diameter of 30 μm and above and with a minimum diameter of 13.5 μm and above, whilst the rest of neurons were considered interneurons.

7.3.3 Determination of myelin status

Using the MBP stain, the myelin status of the VH was assessed. To increase the sample of the examined VHs, partially myelinated VHs were considered either demyelinated or normally appearing VHs according to the proportion of demyelination. The VH was considered demyelinated if the demyelinated area occupied more than 50% of the CSA of the VH; otherwise, it was considered normally appearing VHs. Consequently, VHs were classified into three groups; healthy VHs from controls, normally appearing VHs, and demyelinated VHs.

7.3.4 Correction of neuronal numbers

The resultant neuronal numbers were obtained from counting neurons in cross section areas, ie in two dimensional space. These numbers do not usually reflect the actual number of neurons in the real three dimensional space, especially because the neuronal cell bodies are relatively large and can be counted in serial sections. For accurate estimation of neuronal numbers, stereology that examines serial section is the ideal method.

Since our analysis depends on cross sections and applying stereology is not possible, the smallest visible particle of the neuron, which is the nucleolus, was used to identify the countable neurons. However, size of nucleoli may vary and can be higher than the section thickness. Therefore, the resulted number of countable neurons was corrected using Abercrombie method, which considers section thickness and the particle height [376], which is the nucleolus.

Assuming that the nucleolus is rounded in shape, the height has been estimated by measuring the diameter of all nucleoli in each VH. The mean diameter of all nucleoli in each VH was used to correct the number in that VH. Figure 7.1 shows the distribution of the diameter of the nucleoli. The normal distribution of the nucleolus diameter in Figure 7.1 suggests that there is no significant difference between the diameter of the nucleolus in motoneurons and interneurons.

The used Abercrombie formula for correcting neuronal number:

$$N = \frac{nt}{t + H}$$

Where: N = actual number; n = number of counted particles; t = section thickness; H = particle height.

7.3.5 Statistics

Unpaired t-test was used to compare neuronal counts between MS and controls and between PPMS and SPMS. Multiple linear regression was used to study effects of age, gender, demyelination, disease duration and disease subtype on neuronal numbers and sizes.

Comparison between MS and controls was carried out on all five segments, while comparing PPMS and SPMS was restricted to the upper 4 segments, because there were not enough cases to accomplish reliable comparison in the lower lumbar segment.

7.4 Results

7.4.1 The ventral horn surface area

The surface area of the VHs was reduced in MS spinal cords compared to MS. The reduction in the surface area was significant in the upper cervical ($p = < 0.001$), lower cervical ($p = 0.016$), and upper lumbar segments ($p = 0.006$). There was no difference at the thoracic ($p = 0.934$) and lower lumbar segments ($p = 0.164$).

7.4.2 Quantification of neurons (Control vs. multiple sclerosis)

In controls and MS, the highest number of neurons was found in the VH of the lower lumbar segment. The lowest number of neurons was found in the VH of the thoracic cord. MS showed reduced total numbers of neurons compared to healthy controls. This reduction was more obvious in the higher cord levels. There was a tendency toward significant reduction in the upper cervical ($p = 0.059$), and significant reduction in the lower cervical ($p = 0.024$) and upper lumbar ($p = 0.05$). There was no difference in the thoracic ($p = 0.785$) and the lower lumbar segments ($p = 0.685$).

By considering neuronal types, there was greater loss in interneurons compared to motoneurons, especially in the higher cord levels. For example, in the upper cervical segment, MS cases demonstrated considerable, although not significant, loss in interneurons ($p = 0.059$) with no significant loss in motoneurons ($p = 0.577$). In the lower cervical segment, there was similarly greater reduction in interneurons ($p = 0.045$) compared to motoneurons ($p = 0.094$). Interneurons and motoneurons were both significantly reduced only in the upper lumbar segment (interneurons $p = 0.007$; motoneurons $p = 0.01$). There was no difference between MS and controls in the thoracic (interneurons $p = 0.805$; motoneurons $p = 0.878$) and lower lumbar segments (interneuron $p = 0.371$; motoneurons p value 0.945) (Figure 7.2). The absolute numbers of neuronal counts are mentioned in Table 7.2.

7.4.1 Effect of demyelination on neurons

A reduction was observed in the total number of neurons in the demyelinated VHs compared to controls. All levels showed a tendency toward significance, however, the difference was only significant in the thoracic cord. Compared to controls the p values were as the following; upper cervical (p value 0.073), lower cervical ($p = 0.089$), thoracic ($p = 0.001$), upper lumbar ($p = 0.083$) and lower lumbar ($p = 0.069$).

When the type of neurons was considered, the calculated reduction in number of interneurons in the demyelinated VHs had a trend towards significance in the upper cervical cord ($p = 0.056$). There was no difference in the rest of the cord levels; lower cervical ($p = 0.103$), thoracic ($p = 0.111$), upper lumbar ($p = 0.546$) and lower lumbar ($p = 0.169$). Motoneurons number was not affected in the demyelinated areas compared to normal controls in the upper cervical ($p = 0.972$), lower cervical ($p = 0.242$), thoracic ($p = 0.128$), upper lumbar ($p = 0.066$). However, motoneurons were notably reduced in the lower lumbar ($p = 0.047$) (Table 7.3).

Table 7.2: Comparison between MS and controls in neuronal counts of the ventral horn

Spinal cord segment category	Sample type and number		Total neurons number	Std. Dev	P value	Moto-neurons number	P value	Inter-neurons number	P value
Upper Cervical	Control	n = 6	5.79	3.50	0.059	1.22	0.577	4.56	0.059
	MS	n = 14	3.92	3.14		1.04		2.13	
Lower Cervical	Control	n = 14	8.06	5.09	0.030	3.97	0.094	4.09	0.046
	MS	n = 8	4.07	2.57		2.58		1.95	
Thoracic	Control	n = 2	2.35	0.62	0.785	1.0	0.878	1.36	0.805
	MS	n = 10	2.53	2.16		0.89		1.10	
Upper Lumbar	Control	n = 9	6.50	2.78	0.005	3.35	0.021	3.15	0.007
	MS	n = 12	2.65	1.06		1.49		1.42	
Lower Lumbar	Control	n = 10	9.63	5.07	0.685	5.31	0.945	4.32	0.371
	MS	n = 12	8.66	5.84		5.42		3.24	

Density of neurons was calculated in the demyelinated VHs of MS and was compared with the density from normal appearing VHs and healthy VHs. The mean density of neurons in the demyelinated VHs was 2.45/mm^2 and in the normally appearing VHs was 3.68/mm^2 ($p = 0.003$). Compared to the density of neurons in the healthy VHs, which was 3/mm^2, there was no significant difference from demyelinated areas ($p = 0.200$).

Similar results were obtained when the type of neuron was considered in the analysis of density. Interneurons' density was significantly reduced in the demyelinated VHs compared to normally appearing VHs ($p = 0.021$) but insignificantly reduced compared to normal controls ($p = 0.188$). Similarly, motoneurons' density was significantly reduced compared to normally appearing VHs ($p = 0.024$) and insignificantly reduced compared to health VHs ($p = 0.663$) (Table 7.4).

7.4.2 Quantification of neurons in the normal appearing ventral horns

Total number of neurons was compared between normal appearing areas and normal controls. Apart from the upper lumbar segment, results did not show a difference between demyelinated and controls VHs at any of the cord levels (upper cervical p = 0.126; lower cervical p = 0.095, thoracic p = 0.865; upper lumbar p = 0.004; lower lumbar p = 0.882). This applies to interneurons (upper cervical p = 0.144; lower cervical p = 0.189; thoracic p = 0.948; upper lumbar p = 0.06; lower lumbar p = 0.618). Motoneurons' number did not show a difference between normal appearing and normal controls at any level, except in the upper lumbar segment (upper cervical p = 0.454; lower cervical p = 0.171; thoracic p = 0.828; upper lumbar p = 0.027; lower lumbar p = 0.540) (Figure 7.3).

Table 7.3: Total number of neurons in the demyelinated and normally appearing VHs (The Abercrombie correction)

Spinal cord segment category	Myelin status	number	Corrected Total neurons number	Corrected interneurons number	Corrected motoneurons
Upper Cervical	Demyelinated VHs	n = 7	2.74	1.50	1.24
	Normally appearing VHS	n =16	3.36	2.41	0.95
Lower Cervical	Demyelinated VHs	n =9	4.74	1.95	2.79
	Normally appearing VHs	n =6	4.21	1.94	2.27
Thoracic	Demyelinated VHs	n =6	0.78	0.44	0.34
	Normally appearing VHs	n =12	2.60	1.43	1.16
Upper Lumbar	Demyelinated VHs	n =3	3.05	2.18	0.87
	Normally appearing VHs	n =19	2.89	1.30	1.59
Lower Lumbar	Demyelinated VHs	n =2	2.04	1.01	1.03
	Normally appearing VHs	n =10	9.99	3.69	6.29

Table 7.4: Density of neurons in demyelinated, normally appearing and healthy VHs

Myelin status	Neurons density	Motoneurons density	Interneurons density
Demyelinated	2.45/mm^2	1.17/mm^2	1.28/mm^2
Normally appearing	3.68/mm^2	1.69/mm^2	1.99/mm^2
Normal control	3.00/mm^2	1.26/mm^2	1.74/mm^2

7.4.3 Quantification of neurons in PPMS and SPMS

Comparing the total number of neurons between the two disease subtypes did not reveal a significant difference at any of the cord levels. These results were similar

with interneurons and with motoneurons. There was no difference in the total number of neurons in the demyelinated VHs between PPMS and SPMS (upper cervical p = 0.479; lower cervical p = 0.225; thoracic p = 0.990; upper lumbar p = 0.262). This was also found with interneurons (upper cervical p = 0.570; lower cervical p = 0.115; thoracic p = 0.507; upper lumbar 0.074) and with motoneurons (upper cervical p = 0.509; lower cervical p = 0.462; thoracic p = 0.578; upper lumbar p = 0.498) (Tables 7.5 and 7.6).

Table 7.5: Comparison between PPMS and SPMS in neuronal counts regardless of myelin status

Spinal cord segment category	Type of the disease		Total neurons			Motoneurons			Interneurons		
			value	P *	p **	value	P *	p **	value	P *	p **
Upper Cervical	PPMS	n=14	3.92	0.254	0.102	1.13	0.810	0.435	2.79	0.261	0.121
	SPMS	n=9	2.00	0.045		0.89	0.452		1.11	0.052	
Lower Cervical	PPMS	n=8	4.07	0.024	0.469	2.61	0.135	0.928	1.45	0.021	0.264
	SPMS	n=7	5.05	0.086		2.54	0.155		2.51	0.223	
Thoracic	PPMS	n=10	2.53	0.912	0.157	1.03	0.963	0.509	1.51	0.896	0.163
	SPMS	n=8	1.32	0.184		0.72	0.644		0.60	0.542	
Upper Lumbar	PPMS	n=12	2.65	0.001	0.232	1.30	0.014	0.261	1.35	0.034	0.682
	SPMS	n=10	3.22	0.008		1.72	0.041		1.50	0.090	
Lower Lumbar	PPMS	n=12	8.66	0.685	—	5.42	0.942	—	3.24	0.371	—

P* comparison of the means between the subtype and the corresponding segment from controls
P** comparison of the mean between the two subtypes.

Including all the cord levels, the average density of neurons in the demyelinated VHs of PPMS was 2.5/mm^2 and in SPMS was 2.4/mm^2 (p = 0.882). The density of motoneurons was 1.2/mm^2 in PPMS and 1.2/mm^2 in SPMS (p = 0.959), while the density of interneurons was 1.3/mm^2 in PPMS and 1.2/mm^2 in SPMS (p = 0.726).

Table 7.6: Comparison between PPMS and SPMS in the number of neurons in the demyelinated VHs

Spinal cord segment category	Type of the disease		Total number of neurons		interneurons		motoneurons	
			number	P	number	p	number	p
Upper Cervical	PPMS	n = 4	3.63	0.479	1.86	0.570	1.39	0.509
	SPMS	n = 3	3.50		1.04		1.04	
Lower Cervical	PPMS	n = 6	1.81	0.225	1.37	0.115	2.52	0.462
	SPMS	n = 3	2.15		3.10		3.32	
Thoracic	PPMS	n = 4	1.60	0.990	0.52	0.507	0.27	0.578
	SPMS	n = 2	1.60		0.28		0.50	
Upper Lumbar	PPMS	n = 2	4.85	0.262	2.94	0.074	1.31	0.498
	SPMS	n = 1	1.04		0.65		0.00	
Lower Lumbar	PPMS	n = 2	1.75	—	1.01	—	1.03	—

7.4.4 Sizes of neurons (MS vs. controls)

The CSA of interneurons did not show difference between MS and controls in all levels (upper cervical p = 0.630; lower cervical p = 0.732; thoracic p = 0.830; upper lumbar p = 0.544; lower lumbar p = 0.995). Similarly, motoneurons CSA was not different in MS from healthy controls (upper cervical p = 0.853; lower cervical 0.091, thoracic p = 0.569, upper lumbar p = 0.788; lower lumbar p = 0.681).

7.4.5 Effect of demyelination on neuronal sizes

Within MS cases, there was no significant difference between demyelinated areas and NAGM in interneurons CSA (upper thoracic p value 0.134; lower cervical p value 0.274; thoracic p value 0.238; upper lumbar p value 0.587, lower lumbar p value 0.098). There was also no difference between Motoneurons' sizes in demyelinated and normal appearing areas (upper cervical p value 0.124, lower cervical 0.323, thoracic p value 0.317, upper lumbar p value 0.333; lower lumbar p value 0.298) (Figure 7.3)

7.4.6 Sizes of neurons (PPMS vs. SPMS)

Regardless of myelin status, the size of interneurons was compared between the two disease subtypes in the 4 cord segments. The obtained data did not demonstrate any significant difference between the two forms of the disease (upper cervical p value 0.601; lower cervical p value 0.099; thoracic p value 0.427, upper lumbar 0.056). The only significant difference in motoneurons' sizes was at the lower cervical level (upper cervical p value 0.129; lower cervical p value 0.001; thoracic p value 0.909; upper lumbar p value 0.732).

In the demyelinated areas, there was no significant difference between PPMS and SPMS in CSA of interneurons (upper cervical p value 0.240; lower cervical p value

0.420; thoracic p value 0.216; upper lumbar p value 0.117) and motoneurons (upper cervical p value 0.452; Lower cervical p value 0.026; Thoracic p value 0.376). In normally appearing areas, there was no significant difference between the two forms in sizes of interneurons (upper cervical p value 0.789; lower cervical p value 0.059; thoracic p value 0.962; upper lumbar p value 0.083) and motoneurons (upper cervical p value 0.196; lower cervical 0.145; thoracic p value 0.504; upper lumbar p value 0.955) (Figure 7.3 and Table 7.7).

Table 7.7: Neuronal sizes in the demyelinated, normally appearing, and healthy VHs in the two disease forms

Spinal cord segment category	Myelin status	Mean cross sectional area of interneurons in μm^2	Mean cross sectional area of motoneurons in μm^2
Upper Cervical	Demyelinated	189	633
	Normally appearing	242	762
	Normal control	249	721
Lower Cervical	Demyelinated	283	1156
	Normally appearing	243	992
	Normal control	290	799
Thoracic	Demyelinated	202	724
	Normally appearing	257	864
	Normal control	245	912
Upper Lumbar	Demyelinated	290	1040
	Normally appearing	241	961
	Normal control	238	969
Lower Lumbar	Demyelinated	414	956
	Normally appearing	269	1456
	Normal control	254	1188

7.4.7 Effects of age, gender and disease duration on quantification of neurons

In controls, the total number of neurons is significantly affected by age ($p = 0.005$) and the cord segment ($p = 0.02$) controlling for gender, cord segment, and post-mortem delay. Total neurons were reduced by age and were higher in the lower cord segments. When the type of neuron was considered, it was only the motoneurons that were reduced by age (Correlation -0.805, $p = 0.001$) and were also affected by cord level ($p = 0.014$) and not interneurons (Table 7.8).

Examining effects of independent factors in MS illustrated that the cord level (p value 0.005) is the only factor that has a significant effect on total neuronal number controlling for age, gender, myelin status, duration of the disease, and post-mortem delay. However, controlling for the same factors, spinal cord level has only significant effects on motoneurons' numbers (p value < 0.001) and not on interneurons (p value 0.646). There is also significant effect of gender on number of motoneurons (p = 0.04) and not on interneurons (p 0.524), where motoneurons numbers was greater in males.

Table 7.8: correlation between age and numbers of neurons in controls

Control Variables		Corrected interneurons number	Corrected motoneurons number	Corrected total number of neurons
Gender & Spinal cord segment category & Post-mortem delay	Correlation	-.359	-.805	-.730
	Significance (2-tailed)	.228	.001	.005

7.4.8 Summary of results

In controls, there seem to be a reduction in the motoneurons with age progression. There is also an increase in the number of motoneurons in the lumbar cord compared to the cervical cord.

Compared to controls, total neurons count appeared to be reduced in MS and showed tendency toward significance for this reduction in most of the cord levels. This affected both motoneurons and interneurons. However, it seems that interneurons suffered from greater loss. There was no significant difference between PPMS and SPMS in numbers of interneurons and motoneurons. Demyelination possibly has negative effects on numbers of neurons. There was a reduction in total neurons in the demyelinated areas compared to normal controls, which again affected mainly interneurons. Density of neurons was significantly reduced in the demyelinated VHs compared to normal appearing VHs. There was no significant difference between PPMS and SPMS in numbers of interneurons or number of motoneurons and this applied to demyelinated and normally appearing areas. Our

data did not demonstrate considerable effects of demyelination or NAGM on neuronal sizes. There was no difference between PPMS and SPMS in this respect. Numbers and morphology of neurons probably varies among different spinal cord levels especially motoneurons, while interneurons do not show great variation.

7.5 Discussion

This study might be the first histopathological study that has quantified neurons in PPMS and SPMS in the spinal cord. The results obtained from the study provide additional evidence that there is loss of neurons in the spinal cord GM due to MS with no variation between the two disease subtypes. There was a trend toward significance regarding neuronal loss that mainly affected the upper segments and demyelinated areas with no difference between the two disease subtypes.

7.5.1 Source of errors in neuronal counts

In contrast to a number of previous studies, our study has examined a relatively big sample size [131, 351, 358], but fewer than Gilmore's et al study (MS n = 37; controls n = 22). Loss of neurons in our sample was less extensive than in Gilmore's et al study. This could be due to difference in sample size, section thickness, the stain used, and the method used for calculations (video camera vs electronic images). More importantly, due to lack of enough completely demyelinated VHs in our sample, we considered the VHs that have demyelination of 50% and above as demyelinated. In contrast, Gilmore only considered the completely demyelinated VHs in the analysis.

The study by Gilmore et al quantified neurons within the VH of human spinal cords in multiple cord levels; upper cervical, upper thoracic, and lumbar. Gilmore used Cresyl violet stain on 15 μm thick sections. In comparison, we have used

immunohistochemistry against NE14 to trace neurons on 5 μm thick sections. It is expected that thick sections would include a greater number of nucleoli compared to thinner sections (the split cell error) [377].

Accurate neuronal count has been under debate for a long time. It is estimated that the most precise methods have ± 10% error [377]. In addition, Abercrombie has stated that his correction formula may have up to 10% of overestimation. Another error that may have arisen from using the Abercrombie method is that shape and orientation of cells are not taken into account. In addition, the way that Abercrombie has identified the particle height is not always accurate, because the method assumes that the level of cut is on right angle through the particle (nucleolus in our study) [377]. Furthermore, due to the difference in nature of particles, William et al 1988 has indicated that some cells may be pushed aside during cut (like cutting a mixture of gelatine and hazel nuts with a knife) [377].

Another issue is that the Abercrombie correction formula is sensitive to tissue thickness. This problem may not only apply on different studies, but may affect the same study. For example, it was reported that there is a 50% variation in thicknesses of adjacent paraffin sections [378]. This has been attributed to unstable tissue blocks or knives, or due to temperature changes [377]. Another potential source of error is that the Abercrombie correction formula is biased when the particle height is more than one third of the section thickness, as in our study.

An important concern with neuronal numbers is the presence of significant normal individual variation [379]. There is also normal variation in motoneurons' numbers between different spinal cord segments which has been reported in this study. Table 7.9 demonstrates the variation in neuronal numbers among healthy controls.

Table 7.9: Normal variation in neuronal numbers (numbers were corrected in each ventral horn using the Abercrombie correction formula)

Spinal cord segment category	Minimum (corrected total number)	Maximum (corrected total number)	Mean	SD	Coefficient of variation (SD/Mean)%
Upper Cervical	1.13	10.32	5.79	3.50	60
Lower Cervical	2.54	17.57	8.06	5.09	63
Thoracic	1.91	2.79	2.35	0.62	26
Upper Lumbar	2.92	11.73	6.50	2.79	43
Lower Lumbar	2.79	16.96	9.63	5.07	53

7.5.2 Neuronal counts

With respect to neuronal pathology in the spinal cord, two important findings have been further confirmed in this study; there is a greater degree of neuronal loss in the DMGM of the spinal cord compared to controls and normally appearing VHs. This loss seems to affect interneurons more than motoneurons. It is possible that interneurons are more sensitive to demyelination, and thus more prone to injury, compared to motoneurons [106]. This can be also due to possible size selective neuronal injury which can be due to retrograde degeneration caused by the size selective axonal loss [95].

Similar to other elements, such as OLs and axons, it seems that there is also predilection for upper cord segments with respect to neuronal injury. Greater loss of neurons in the upper segments has been reported by Gilmore et al, and was attributed to a high proportion of demyelination in upper segment compared to lower segments. The relationship between demyelination and loss of neurons has also been reported in other sites of the CNS. There was 30% loss of neurons in the demyelinated regions of the hippocampus [362] and 10% loss in the demyelinated regions of the cerebral cortex [358].

Demyelination may result in loss of neurons by depriving neurons of trophic support. Trophic support of neurons may come from neuroglia or from myelin [380-385]. Within the GM, neurons have an intimate relationship with perineuronal OLs. A

previous electron microscopic study demonstrated the presence of a sheath of myelin-like substance surrounding some neuronal bodies in the human cerebral cortex [169]. Therefore, one of the presumed functions of perineuronal cells is supporting neurons. There is evidence that perineuronal OLs may protect neurons from undergoing apoptosis [386]. In previous chapters, we demonstrated excessive loss of OLs in the spinal cord DMGM. Loss of these OLs may promote neuronal apoptosis.

In this study there was no reduction in neurons in the normally appearing VHs. While loss of neurons in NAGM has been reported in the thalamus [131] and the lateral geniculate nucleus [95], other studies reported normal neuronal counts in the NAGM of the cerebral cortex [351, 358] and the spinal cord [351]. It seems that neuronal pathology is not similar between different regions of the GM in the CNS. Recent studies reported variation in GM pathology according to the region involved [129, 387, 388].

A possible explanation is that inflammation seems to be different between various GM regions. There is little inflammation in the cerebral cortex lesions compared to the WM lesions [389]. In comparison, deep GM nuclei inflammation score was intermediate between degree of inflammation in the WM and that of the cortical GM [388]. In comparison, the spinal cord GM did not show a difference in inflammation from the nearby WM [124].

Another explanation is that the degree and nature of GM demyelination is different between GM regions. In the cerebral cortex, it has been suggested that MS starts as focal disease in the WM and that cortical demyelination is part of chronic progression [390]. On the other hand, deep GM nuclei pathology appears to be different from

cortical GM pathologies. Vercellino et al 2009 studied deep GM pathology in 14 cases of MS. Demyelination affected mainly the thalamus and caudate, which are the areas of excessive connections. The spinal cord GM demyelination seems to be different from the cerebral cortex and the deep GM nuclei. This is because spinal cord lesions are commonly mixed GM and WM lesions [124].

Another possible reason is that the cerebral cortex, deep GM nuclei, and the spinal cord GM have different connections with various parts of the CNS. It is expected that areas with more extensive connections may undergo degenerative neuronal loss in the NAGM more than other parts. It is important to differentiate between two types of connections in the CNS; distant connections with various parts of the CNS via long axons, and local connections via interneurons. It is possible that areas with multiple distant connections are more vulnerable to retrograde degeneration than areas with local connections only. For example, the thalamus is connected to many regions of the CNS with long axons [212]. Tao et al 2009 conducted a MRI study on the deep GM nuclei. The thalamus and caudate showed the highest degree of atrophy. Interestingly, deep GM nuclei atrophy correlated significantly with WM lesions. Such a finding further supports the assumption that pathology of deep GM nuclei (unlike pathology of the cortical and spinal GM which seems to be independent of WM lesions) depends considerably on WM lesions [129].

A number of studies support the effects of long term Wallerian and transynaptic degenerations on GM by pathology study [95] and MRI study [94]. Pathologically, Evangelou demonstrated loss of small neurons in the NAGM of the geniculate nucleus secondary to loss of axons in the optic tract. Radiologically, Sepulcre et al 2009 studied the contribution of different WM lesions in causing lateral geniculate nucleus atrophy in 61 MS patients. Using MRI, lateral geniculate nucleus atrophy

was measured and mapping the WM was carried out. Lateral geniculate atrophy correlated with lesions of the optic tract and not with any other lesions [391]

Our study did not reveal changes in neuronal numbers in the NAGM. This may indicate that the role of retrograde degeneration is limited in causing neuronal injury of the normal GM of the spinal cord. In the VH neurons are involved in a complex network of local interneurons that maintains circuits of action potentials, and this may prevent or delay degeneration of neurons with severed axons.

7.5.3 Correlation between loss of neurons and atrophy of the GM

In normal controls, the relationship between the volume of the GM and the number of neurons is already established in the spinal cord. Normally, there is greater amount of GM in the thickened spinal cord segments. The VHs of the lower cervical and lower lumbar have greater volume because they have high numbers of neurons to supply the upper limbs and the lower limbs.

To our knowledge, one previous study has demonstrated significant correlation between neuronal loss and atrophy and this was in the archaecortex [362]. The main constituent of cortical GM atrophy is demyelination of axons and to a lesser extent neuro-axonal loss, especially in the frontal cortex [216]. In this study, we reported atrophy of the VHs which affected mainly the higher cord segments. There was also significant correlation between number of neurons and surface area of the VH. Therefore, it is possible that one of the main constituents of spinal cord GM atrophy is neuronal loss.

7.5.4 Interpretation of results from neuronal sizes and source of error

We used size criteria to differentiate between motoneurons and interneurons. Using fixed size criteria may produce variable results because of different shrinkage factors among different types of tissues. In addition, neurons themselves may interact differently to fixation process, ie neurons may shrink more than the nearby neuropil. This is suggested from the common profile in Figure 7.4. It has been also suggested that shrinkage is not uniform throughout the section, because shrinkage near the section centre may be greater than shrinkage at the section edge [392].

However, we should acknowledge that classification of neurons, depending on maximum and minimum diameters, may affect interpretation of results obtained from neuronal sizes. Although measurement of neuronal sizes was independent of the minimum and the maximum diameters, classification of neurons into interneurons that are less than 13.5 μm minimum width or less than 30 μm maximum diameters may reflect neuronal sizes. Moreover, assuming there is considerable neuronal shrinkage in MS; motoneurons may shrink to be within interneurons determined range. Additionally, it is not accurate to apply the same range of diameters classification of neurons on MS and controls when there is a possibility of neuronal atrophy in MS.

Nevertheless, the NE14 stain does not differentiate between motoneurons and interneurons; therefore, we had to use size criteria to differentiate between the two subtypes. Size criteria has been used in previous studies [375, 393-396]. But in those studies, discrimination was sometimes associated with morphological observation to differentiate between motoneurons and interneurons. Only the study by Gilmore employed size criteria without considering morphological variation [106].

Because of the irregular shape of neurons and the fact that we count them on cross sections that can pass any level of the neuronal cell body, morphological discrimination between motoneurons and interneurons is highly subjective. Therefore, considering maximum and minimum diameter of the neuron may be a good indicator of the type of neuron, especially with the minimum diameter, as the minimum diameter of an object does not change with the level of cut. This fact is based on the assumption that level of cut is through the centre of the cell body, which is the nucleolus in our example. In addition, neurons are very irregular in shape and effects of maximum and minimum diameters on neuronal size are possibly minimal.

In the present study, there was no significant difference between neuronal sizes in controls and MS cases and between PPMS and SPMS. Gilmore et al demonstrated significant reduction in sizes of interneurons which affected demyelinated and normal appearing areas. Neuronal shrinkage has been reported also in the hippocampus [362]. Gilmore et al reported increase in sizes of motoneurons which has been attributed to size selective neuronal loss, misinterpretation of shrunken small motoneurons as interneurons, or pathological swelling of motoneurons. In comparison, in our study there was no significant difference between sizes of motoneurons in MS and controls.

7.5.5 Quantification of neurons PPMS vs. SPMS

The relationship between neuronal loss and clinical course of the disease in PPMS and SPMS seems to be a complex issue, owing to complexity of neuronal circuits and variation between ascending and descending tracts in nature and speed of neuronal degeneration.

While correlation between spinal cord GM pathology and disability has not yet been established, correlation between brain GM (cortical and deep GM nuclei) and disability has been reported. A study has investigated the correlation between deep GM nuclei pathology and clinical progression of the disease in 97 patients. It was stated that there is strong association between deep GM lesions and worsening of symptoms [128].

The correlation between axonal loss and disability has been reported several times [397-399], but the clinical significance of neuronal loss specifically has not been demonstrated yet For example, despite having a greater degree of demyelination and neuronal loss in the cervical segment of the spinal cord, upper limbs' paralysis is not a recognized feature of MS compared to other motoneurons diseases. Additionally, in previous chapters, we demonstrated differences in demyelination, atrophy, OLs and axonal loss between PPMS and SPMS. In this study, SPMS showed a tendency toward fewer numbers of neurons compared to PPMS, but the difference was not statistically significant.

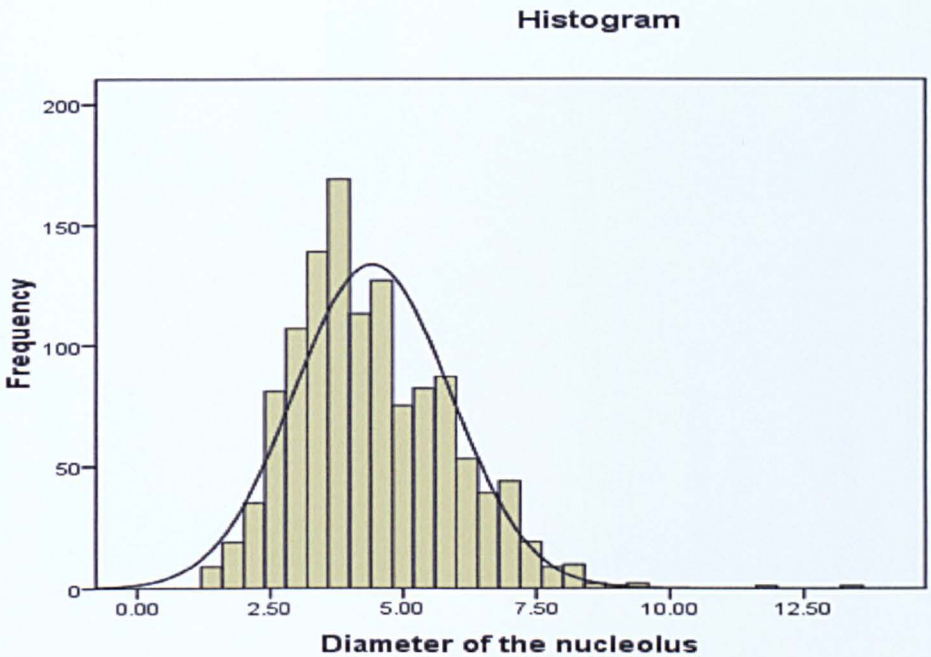
This is possibly because number of neurons is significantly less than number of OLs and axons in a cross section. Therefore, a potential difference between PPMS and SPMS in the amount of neuronal loss may be minimal and thus a greater sample size is needed to reveal such a difference. It is also possible that surviving neurons are not always functioning and this affects results obtained from correlation with disability.

7.6 Conclusion

In summary, the study has further confirmed the presence of neuronal loss in the demyelinated GM of the spinal cord with no remarkable loss in the NAGM [106, 400,

401]. Demyelination seems to have significant effect on neuronal loss possibly by causing loss of trophic support from neuroglia and/or neurotoxicity from molecules released within the plaque. Despite the presence of OLs in GM lesions of PPMS and SPMS, numbers and function of these OLs may be insufficient to protect neurons [236, 238, 239]. In addition, neuronal loss was found to be an early processes [357].

Neuronal loss was found to be greater in the upper cord segments [106]. This can be associated with demyelination that affects mainly the upper segments. Unlike neuronal loss in the thalamus or geniculate nucleus, neuronal loss in the spinal cord GM is probably due to local lesion factors and independent of distant lesions. This is evident from the observation that neurons are spared in the NAGM. More importantly, despite differences between PPMS and SPMS in degree of demyelination, atrophy, OLs loss and axonal loss, there was no significant difference in numbers of neurons.



Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation	Skewness		Kurtosis	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
Diameter of the nucleolus	1224	1.27	13.30	4.4201	1.46328	.724	.070	1.176	.140
Valid N (listwise)	1224								

Figure 7.1: Distribution of the diameter of the nucleoli in the neurons of the ventral horn. The normal distribution may indicate that there is no significant difference between the diameter of the nucleoli of the motoneurons and the interneurons. A significant difference would probably produce bipolar distribution

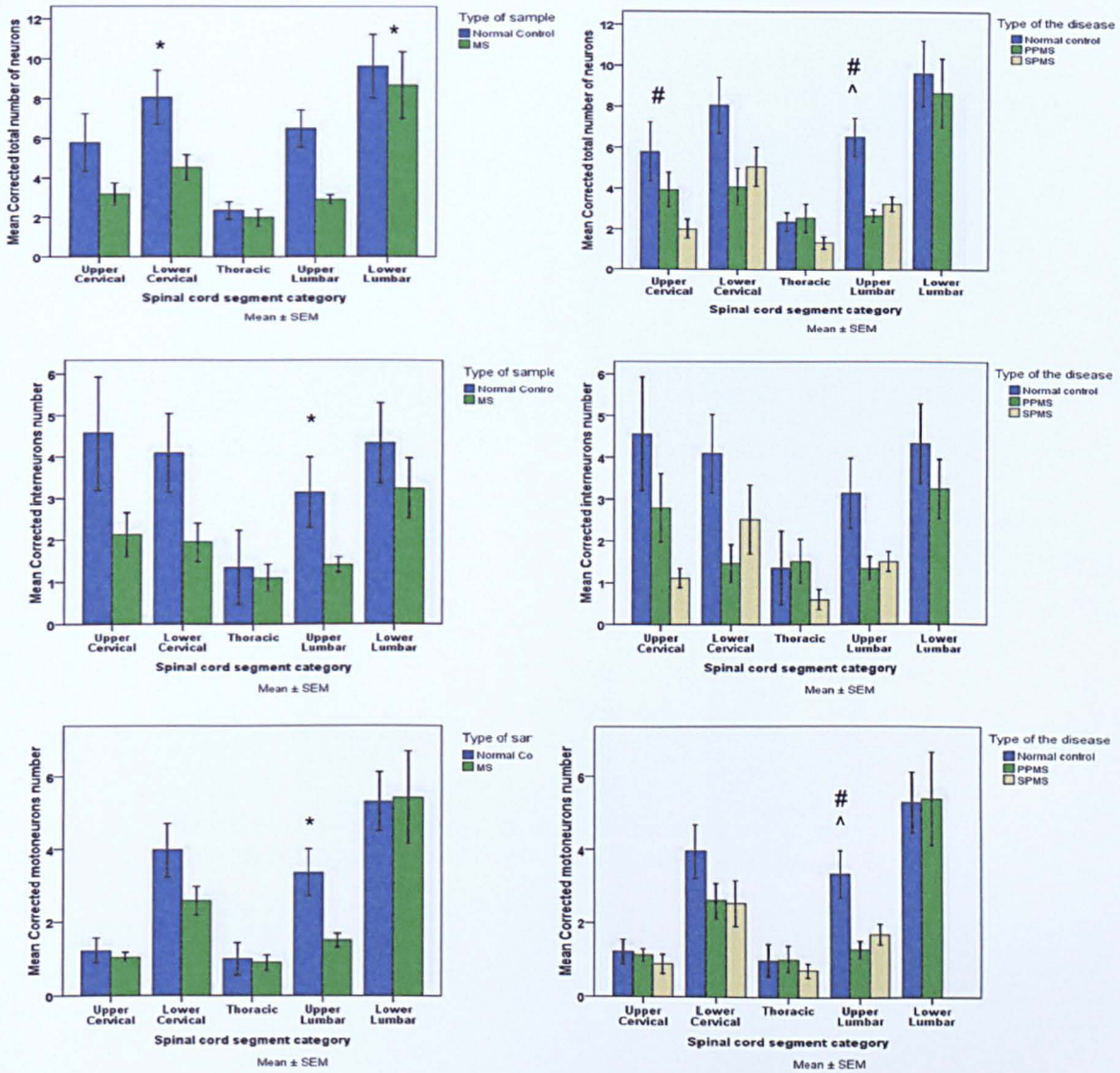


Figure 7.2: The Left bars illustrate the total number of neurons, number of interneuron, and number of motoneurons in the 5 cord segments of MS and controls. Note that there is a greater reduction of neurons in the upper cord segments and this affects interneurons more extensively. The right bar chart illustrates the total number of neurons, interneuron, and motoneurons in the 5 cord segments in PPMS, SPMS and in controls. SPMS and PPMS have no significant difference between each others in neuronal numbers. The Data represent the mean corrected number of all neurons, interneurons, and motoneurons ± SEM. The significant difference between MS and controls are indicated by asterisks, the significant difference between controls and SPMS are indicated by the number sign #, while the significant difference between PPMS and control are indicated by ^. The p values < 0.05 are indicated by one sign, while the p values < 0.01 are indicated by two signs.

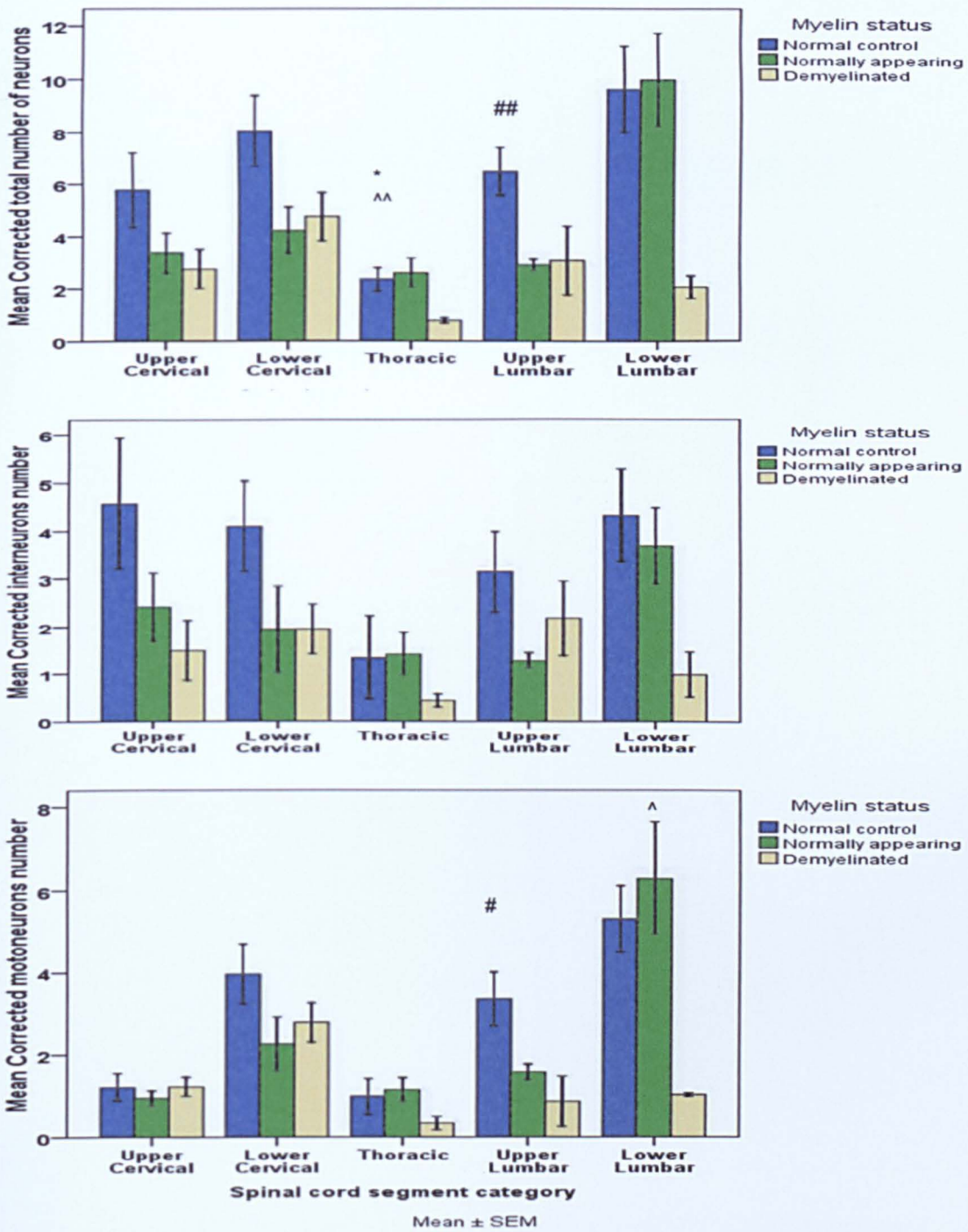


Figure 7.3: A bar chart showing number of neurons, interneurons, and motoneurons in the five cord segments in normal controls, normally appearing VHs, and demyelinated VHs. The bars represent the mean number of neurons, internerons, and motoneurons in the VHs ± SEM. The significant difference between normally appearing VHs and demyelinated VHs are indicated by an asterisk *, the difference between normally appearing VHs and controls are indicated by the number sign #, while the significant difference between normal controls and demyelinated VHs is indicated by ^. The significant differences of p values < 0.05 are indicated by one sign, while the p values < 0.01 are indicated by two signs.

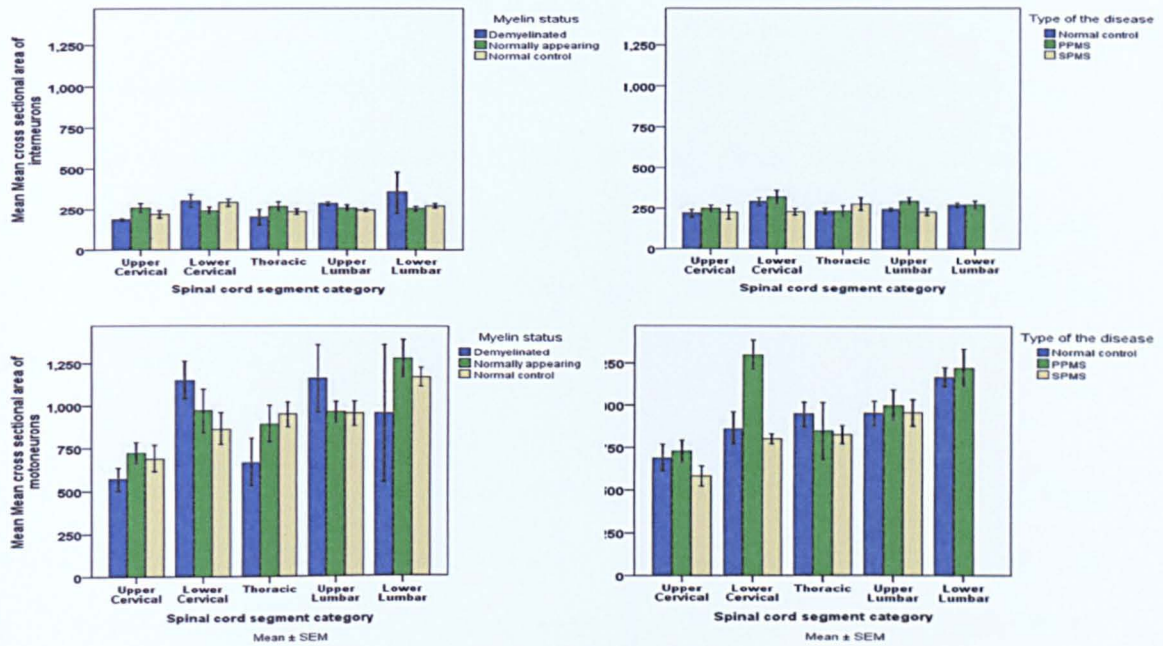


Figure 7.4: A bar chart shows the mean cross sectional areas of interneurons and motoneurons in the demyelinated, normally appearing and healthy GM and in the two disease subtypes. Data represent the mean CSA of neurons and motoneurons ± SEM. There was no significant difference between PPMS, SPMS and controls in the CSA of neurons.

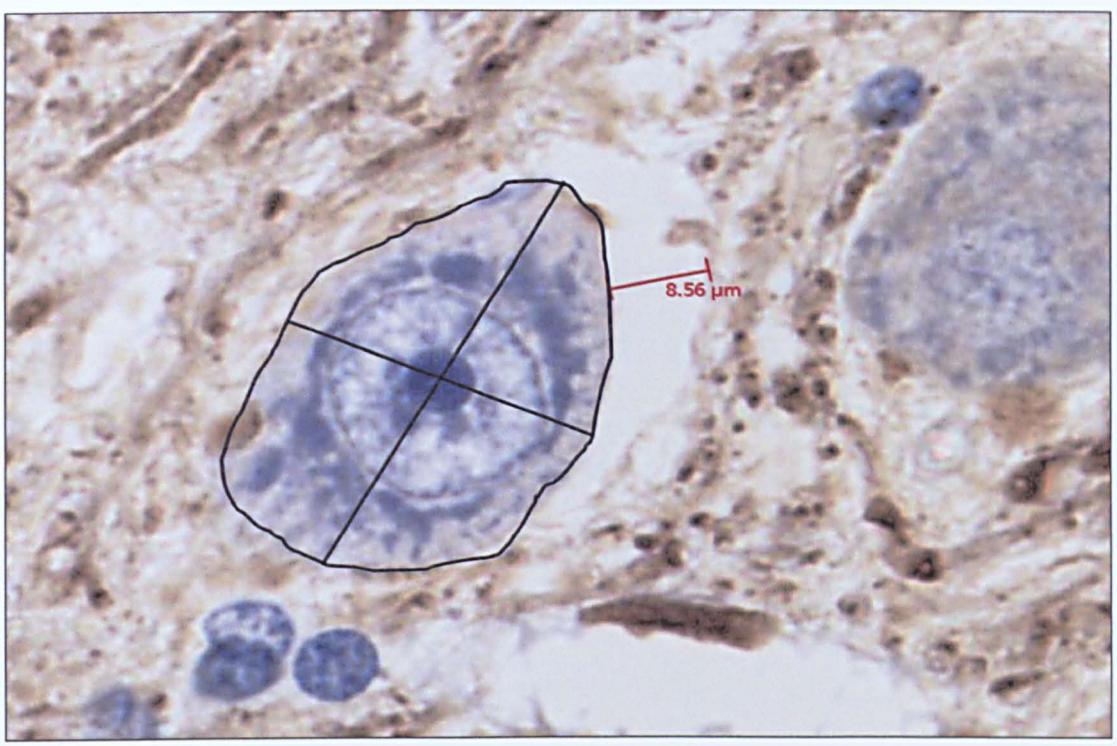


Figure 7.5: A neuron in a human VH (a common profile in our study). Note the space between neuropil and the boundary of the neuron. This can be due to variation in post-mortem changes, during fixation and tissue processing

Chapter 8: Summary and conclusion

The two subtypes of progressive MS have different clinical course at the early stages of the disease. SPMS begins with relapsing remitting stage that may continue for many years, while PPMS is progressive from the beginning. Many believe that with better understanding of the disease pathology, more effective treatment can be achieved in the future.

In this study, the two examined groups of tissue (SPMS and PPMS) derived from patients that had similar degree of disability prior to their death. The study has focussed on quantifying the major five pathological changes associated with PPMS and SPMS. These changes include the lesions load, tissue atrophy, OL, axons and neuronal pathology.

We have chosen the spinal cord for this comparison for a number of reasons. The spinal cord anatomy allows for comparing similar anatomical regions in the two examined groups. The spinal cord has both ascending and descending tracts that may be affected by the same lesion. Demyelination of the spinal cord commonly affects the GM and the WM within the same lesions. Furthermore, the spinal cord lesions are frequently associated with clinical symptoms and thus may explain the clinical variation between the two subtypes [127].

One of the characteristic features of MS is the presence of demyelination preferentially affecting different regions of the CNS. The study has addressed the topographic distribution of demyelination throughout the spinal cord in the two subtypes. The demyelination was found to be greater in the upper cord levels in both forms of the disease. However, it appeared that SPMS spinal cords were affected by a higher degree of demyelination compared to PPMS especially in the upper cord

levels. Another important feature of progressive MS is tissue atrophy. There is obvious atrophy in SPMS and PPMS spinal cords. This has again affected mainly the upper levels of the spinal cords. When the volumes of the spinal cords were compared between the two groups, SPMS showed more atrophy especially in the upper levels. It is possible that due to the presumed higher lesion load and frequency of occurrence of new lesions, SPMS may cause atrophy faster than PPMS

There seems to be correlation between lesion load and atrophy of the spinal cord; demyelination and atrophy affected mainly the upper cord levels and both were greater in SPMS. We found significant correlation between demyelination and atrophy in SPMS but not in PPMS. Therefore, it is possible that lesion load plays a more important role in atrophy of tissue in SPMS compared to PPMS.

Quantification of OLs in the GM and the WM has been carried out in this thesis. Number of OLs was found to be low in the demyelinated areas of the WM and the GM. Although SPMS demonstrated higher number of OLs in the demyelinated areas compared to PPMS, the presence of these OLs was not associated with myelin in the two disease forms. This is possibly because their number is very low in the two subtypes compared to the normal controls or to the nearby normally appearing areas. In addition to low numbers of OLs in chronic lesions, it is possible that these OLs are immature and unable to myelinate.

Outside the lesion, there was a surprising increase in OLs numbers in the NAGM compared to normal controls and the NAWM. This was observed particularly in SPMS. This could be due to presence of extra sources of OLs in the GM or the abundance of inhibitory factors on OLs proliferation in the WM, such as myelin and

dystrophic axons. In addition, SPMS may maintain higher inflammation and thus induce more proliferation of cells including OLs.

Axons are also damaged in MS. Axonal loss affected the DMWM with no difference between the two subtypes in the density of axons. There was also no difference in the density of axons in the lesions of the CST and the PCP. This may indicate that the local effect of demyelination on axons does not depend on the disease subtype or the affected tract. The difference in axons' density between the two tracts and the two subtypes was apparent in the NAWM. There was considerable loss of axons in the NAWM of the descending tracts compared to the ascending tracts. There was also greater loss of axons in the NAWM of SPMS compared to PPMS. It is possible that the mechanism of axonal loss in the NAWM is a different from that in the DMWM. Loss of axons in the NAWM can be due to the effect of Wallerian degeneration, while loss of axons in the demyelinated areas is possibly due to both demyelination and degeneration. Wallerian degeneration of the NAWM of the descending tracts is expected to come from higher lesions in the brain where the SPMS consistently showed a greater degree of demyelination, while Wallerian degeneration of the ascending tract is expected to come from caudal levels in the spinal cord where lesions are rare. Consequently, we observed limited loss of axons in the NAWM of the ascending tracts. Another important observation that suggests different mechanism of axonal loss between the DMWM and the NAWM is that there was a very low OLs to axons ratio in the DMWM compared to normal controls while there was normal ratio in the NAWM. This suggests that the oligodendrocytes may play a lesser role in the loss of axons in the NAWM.

The total number of neurons was reduced in MS and showed a tendency toward significance in most of the cord levels. There was greater loss of interneurons

compared to motoneurons. This reduction affected the demyelinated GM but not the NAGM. There was no significant difference between PPMS and SPMS in numbers of interneurons or number of motoneurons and this applies to demyelinated and normally appearing areas. Motoneurons possibly tolerate demyelination better than interneurons. Preservation of neurons in the NAGM is possibly due to the limited effect of retrograde degeneration on the neuron cell body. Neurons of the NAGM of the spinal cord may not be liable to the effects of retrograde degeneration as the case of neurons in other parts of the CNS (for example the thalamus) because of differences in connectivity.

8.1 Conclusion

There are differences between the two disease subtypes in the degree of demyelination, atrophy, OLs density, and axonal density. There was no significant difference in the degree of neuronal loss or axonal loss within the WM lesion itself. Spinal cord axonal pathology is probably responsible for much of the disability in MS, while neuronal loss may have a limited role in causing disability. This is suggested by three main observations; neuronal loss is restricted only to the demyelinated areas, degree of neuronal loss is less than axonal loss, and flaccid paralysis is not a common clinical feature of MS.

The question whether PPMS and SPMS are two different disease entities or not is still debated. Developing RRMS or PPMS from the beginning may be affected by the nature of the disease itself, age of patient at disease onset (PPMS patients are older and thus may have less nervous tissue plasticity), anatomy of the affected tract and degree of decussation (less decussation means more severe symptoms), strategic location and size of the first lesion (initial lesions of PPMS commonly involve

strategic areas such as the brain stem), and probably other genetic or environmental factors [110].

In SPMS, a possible explanation of progression after a period of RRMS is failure of the CNS compensatory mechanisms [402, 403]. This is supported by the fact that high disease activity and frequency of relapses shortens the duration after which RRMS becomes SPMS. It was reported that patients with MS demonstrate a variable duration of time to reach an EDSS of 4, while progression from 4 to 7 takes a similar duration in all patients [41].

8.2 Future work

Many scientists believe that with suitable intervention MS lesions can undergo repair. Such repair can be in the form of remyelination, neuronal preservation or regeneration, and restoring axonal conduction [404]. Inducing repair of spinal cord lesions may be more beneficial than that of brain lesions as these lesions were more associated with clinical symptoms. Spinal cord lesions are also more accessible than brain lesions for introducing molecules or transplanting OLs.

It is important initially to identify the most potent and most appropriate mitogen that is responsible for OLs proliferation. In vivo molecular imaging of the microglia in MS has been carried out, which carries promising results to provide imaging of other cells, especially OLs [405]. Comparing the degree of expression of OL mitogens between PPMS and SPMS and between the GM and the WM may identify the most potent factor that can induce remyelination in MS. This may also explain two important issues; why SPMS demonstrated greater numbers of OLs in the GM areas compared to the corresponding areas in PPMS; and why the NAGM showed greater numbers of OLs compared to the NAWM.

Oligodendrocytes in the NAGM and the DMGM have not been studied in details yet. CA II positivity in OLs is not enough to reveal their level of maturity as CA II is expressed in most developmental stages of OLs. Identifying OLs around GM lesions using double labelling and staining NG2 positive cells may answer the question about origin and function of these cells. Future studies may double stain CA II positive cell with other markers, such as MOG for mature OLs, NG2 for OLs progenitors, PDGFR-α for migratory OLs progenitors, or nestin for multipotent neural stem cells.

It is also important to explore more the relation between lesion load in the brain and the degree of axonal loss in the spinal cord, especially the NAWM of descending tracts. This may explore the mechanism of axonal loss in the spinal cord.

Future studies may include RRMS cases to compare with PPMS and SPMS. The positron imaging tomography for molecular imaging can be used in vivo to compare these subtypes. This will overcome the lack of post-mortem RRMS samples and differentiate between RRMS, SPMS, and PPMS. Including RRMS may explain why, when and how RRMS enters the stage of SPMS. Answering these questions may hopefully lead to treatment that delays relapses or reduces their severity. Such treatment may include introducing agents (systemic or intrathecal) that induce migration and proliferation of OLs to the site of lesions, and transplanting cells in the site of lesions that may have similar characteristics to OLs progenitors [244].

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Appendix A

Station	reagent	time
1	10% formalin	1
2	50% alcohol	3
3	70% alcohol	4
4	95% alcohol	4
5	100% alcohol	4
6	100% alcohol	4
7	100% alcohol	4
8	100% alcohol/100% chloroform (50 / 50 mix)	6
9	100% chloroform	18
10	100% chloroform	6
11	wax	2
12	wax	2
13	wax	6

Immuno Protocol

Slides were put in a beaker of 10mM EDTA (3.7g EDTA in 1ltr distilled water, pHed to 8 with NaOH)
The EDTA was heated in the microwave at power 8 for 25mins
The slides were transferred to cold running water for 15mins
They were incubated in Buffer 1 for 2 x 5mins
They were incubated in 2% normal goat serum for 30mins
They were incubated in 0.5% aqueous hydrogen peroxide for 10mins
Primary antibody was added at 1:8000 made up in DAKO diluent, for 1 hour
They were washed with Buffer 1
They were incubated in Buffer 1 for 2 x 5mins
Secondary antibody was added for 30mins
They were washed with Buffer 1
They were incubated with Buffer 1 for 2 x 5mins
DAB was added for 5 mins
They were was with distilled water
They were counterstained with Haematoxylin
They were dehydrated and mounted with DPX

Buffer 1

1ltr PBS
10g Bovine Serum Albumin
2ml Tween 20

Primary Antibody - Rbt pAb to Carbonic Anhydrase II (Abcam ab6621-5)

Secondary Antibody - part of Envision DAKO K5007 DAB kit

DAB - Provided by Des Powe