A HISTOPATHOLOGICAL AND MAGNETIC RESONANCE IMAGING ASSESSMENT OF MYELOCORTICAL MULTIPLE SCLEROSIS: A NEW PATHOLOGICAL VARIANT

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to Kent State University in partial
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Chapter 1

Section 1: Introduction

Multiple sclerosis (MS) is a chronic immune-mediated demyelinating disease with underlying neurodegeneration of the human central nervous system, which affects over 2.3 million individuals worldwide and currently has no cure. This disease affects 2 times more females than males and is the major cause of non-traumatic neurological disability in young adults, with onset occurring often in the third decade of life (average age 25-33, (1), although pediatric MS also occurs in those 18 years old and younger). The etiology of MS involves a complex relationship between environment and genetic susceptibility. The environmental factors that have been proposed to play a role in MS etiology and risk range from (A) sunlight exposure which is also highly connected to vitamin D status, with an increased prevalence of MS for individuals residing further from the equator; (B) smoking; and (C) infections from viruses like Epstein - Barr virus and human endogenous retrovirus-W. Although these factors seem to play a role in the risk for developing MS, how they actually affect different aspects of the disease, including the onset or course of the disease, is largely unknown. Genetics also play a role in susceptibility to this disease, as demonstrated by the fact that the risk of developing MS increases in those individuals with a family history of the disease, particularly in immediate family members (2). Twin studies have demonstrated an increased concordance rate of 31% in monozygotic twins, compared to 5% for dizygotic twins (3). A specific allele has been identified
as carrying a risk of 16-60% chance of developing MS (4). The gene is for the Human Leucocyte Antigen (HLA) Class II locus, which is part of the major histocompatibility complex located on chromosome 6p21 and has been shown to have a dose effect of increasing disease risk (5). MS patients that were homozygous for the HLA-DR2 allele were more likely to have a severe disease outcome than any other haplotype (5). The HLA-DR2 gene plays a role in antigen presentation on immune cells, and therefore is presenting fragments of myelin proteins to T lymphocytes and controlling their reactivity to myelin. This finding provides strong evidence that MS is an autoimmune disease (6).

All of these factors, both environmental and genetic, likely interact in a complex manner for each individual in slightly different ways. These interactions orchestrate the age of onset, specific symptoms, pathological locations, and clinical course of the disease for each person suffering from MS. Therefore, the unique factors and how they interact with one another leads to the large spectrum of MS variants. Several variants of MS have been described with different clinical, radiological, and pathological phenotypes, but the hallmark of all MS variants is demyelinating white matter lesions, particularly those observed in the cerebrum. A more extensive literature review of MS can be found in Chapter 2 of this work, in which I discuss the history of diagnosis of the disease, the differences between each of the many variants, MS treatments, and the use of magnetic resonance imaging (MRI) in MS. I also provide a description of central nervous system pathology.

Although demyelinating white matter lesions are the pathological hallmark of MS, and MS is generally considered to be a disease of the white matter, the cerebral gray matter, spinal cord and brainstem can also become demyelinated and contribute to the neurological disability of MS patients. Cortical demyelination has been estimated to develop in over 90% of MS patients,
and has been shown to begin developing early in the disease course (7). Cortical demyelination can even exceed white matter lesion loads in the cerebrum in some patients (8). Cognitive deficits and some of the neurological disabilities which accumulate over the course of the disease may be a result of cortical and/or hippocampal demyelination frequently observed in individuals with MS. It is unknown, how cortical lesions arise, as well as how much secondary degeneration of axonal fiber tracts within cerebral white matter lesions contributes to the formation of cortical lesions. Parsing the mechanisms of cortical and white matter lesions would require examining individuals with compartmentalized demyelination to determine the relationship between these two lesions types without the influence of other pathologies. Case reports of patients in which pathology was limited to the spinal cord (9) and others with only cerebral white matter demyelination, but no gray matter lesions, can provide insight into the mechanism of cortical demyelination in MS patients. Some MS patients exhibit strictly spinal cord demyelination, supporting the possibility that spinal cord and cerebral white matter demyelination occur through different mechanisms. Furthermore, if cases of MS patients can develop white matter demyelination in the absence of demyelination of gray matter, would suggest different pathological mechanisms. This variation leads to the question of whether spinal cord and cerebral gray matter demyelination could arise from parallel mechanisms?

Another population variant of MS patients that will be described here, are those who have typical clinical histories and brain MRIs predictive of classical demyelinating white matter lesions, but pathologically lack cerebral white matter demyelination. Despite the lack of white matter demyelination, these patients have accumulated clinical disability and demyelination elsewhere in the central nervous system, including in the spinal cord and cerebral gray matter. This new pathological variant of MS is the focus of this dissertation and will be referred to as
“myelocortical MS.” The objective of this thesis is to elucidate pathologies that result in extensive clinical disability in the absence of white matter lesions. My studies are focused on examining and characterizing this unique population of MS patients which are essentially devoid of cerebral white matter lesions and assess the specificity of MRI for the detection of this type of MS pathology.

Myelocortical MS patients who developed very few, if any, macroscopic cerebral white matter demyelinating lesions upon examination of postmortem coronal brain slices were characterized and evaluated for spinal cord and cortical demyelination as well as other pathologies to determine whether cortical demyelination can occur in the absence of cerebral white matter demyelination. I hypothesized that these myelocortical MS patients would have spinal cord demyelination similar to age (at time of death)- and disability- matched classical MS patients with cerebral white matter lesions since many of the patients from both groups were wheelchair-bound and spinal cord demyelination greatly affects ambulation. In addition, several of these MS patients had also reported cognitive impairments. Thus, I hypothesized that the myelocortical MS patients would have similar or greater amounts of cortical and hippocampal demyelination as those in the classical MS patients. Furthermore, MRI-defined cerebral white matter lesions in these myelocortical MS patients were investigated to determine what underlying pathology was sufficient to result in the abnormal white matter changes in MRI signal in the absence of demyelination. Edema from disruptions in the blood-brain barrier, as well as reactive gliosis and axonal pathology, were hypothesized to have produced these abnormal MRI signals in the white matter. In addition to the above mentioned studies, brain volume loss and the volumes of white matter hyperintensities on MRI scans were measured and compared to the classical MS patients in order to identify any differences in overall damage occurring in these
two MS cohorts. I anticipated that the classical MS patients would contain greater volumes of T2 white matter hyperintensities as well as greater amounts of white matter and whole brain atrophy, measurements due to the increased amount of white matter demyelination in these patients compared to the myelocortical MS patients.

The methodological techniques performed in all of these analyses, such as tissue collection and fixation, immunohistochemistry and various types of quantifications, and statistical analyses are described in Chapter 3 of this thesis. The results from appraising the pathological and MRI characteristics of the myelocortical MS patients in comparison with classical MS patients’ pathology and MRI characteristics are reported and described in detail in Chapter 4. Conclusions about the possible mechanism(s) involved in the pathologies present in myelocortical MS patients versus those present in classical MS and how this relates to MS disease pathogenesis in general are discussed in Chapter 5, as are the strengths, and weaknesses of the study and recommendations for future investigations of this data and myelocortical MS patients.
Chapter 2

Section 5.1 Multiple Sclerosis: General Background

5.1.1 Diagnosis Criteria of MS

Multiple sclerosis was initially described as a neuropathological disease involving demyelinating lesions of the human central nervous system in 1837 by Sir Robert Carswell (10). Several neurologists would follow Dr. Carswell and describe the presence of these demyelinating lesions within the spinal cord, brainstem, cerebellum, and cerebral white matter as gray-to-yellowish brown substances that were hard, semitransparent, frequently centered around blood vessels, and atrophied. It was not until 1866, that Jean Martin Charcot would elegantly describe the symptoms and anatomy of this disease, in addition to naming it le sclerose en plaques (multiple sclerosis, (11)). Charcot introduced a triad of symptoms for this disease, which included intention tremor, scanning speech, and nystagmus, an involuntary eye movement. Furthermore, Dr. Charcot observed three different variations of MS: a form that was cephalic, a spinal form, and a mixed cerebrospinal form (10). This pathological classification of MS did not remain in fashion; MS today is classified most predominantly by clinical history of the disease and with radiological evidence by magnetic resonance imaging (MRI).

William Moxon reported on the disease as having its onset in one’s prime of life (20-45) and that an early remitting stage was often present in patients containing disseminated sclerotic
plaques (10). In this way, he essentially described the major MS subtype known as “relapsing-remitting MS (RRMS)”, which is discussed in greater detail below. In 1916, James Dawson would describe the damage to the myelin and blood vessels, with extensive inflammation within and surrounding the plaques (10). Autoimmunity became the focus of MS disease pathogenesis, of MS, a dogma that still remains one of the leading theories today. Since these initial observations of MS, criteria for the diagnosis of MS have been established and revised to include many variants of the MS spectrum, while excluding many neurological diseases that mimic the symptoms, but not the pathology of MS.

Even with Dr. Charcot’s triad of symptoms, eventually neurologists such as Sydney Allison and Harold Millar, took the criteria a step further, specifying that the patients should have a history of remission and evidence of scattered lesions at different levels in the central nervous system. They also categorized cases based on their histories as to how likely they were to be MS; the first type was early MS, which included patients who did not yet have a remitting history, but had symptoms commonly associated with the disease. McAlpine developed the naming criteria of Probable and Possible MS. Probable MS referred to when patients had physical disability and a history of remissions. The patients who did not fit these first two types could have fallen under the Possible MS category, if their history was progressive, static, or if evidence for lesions at different levels of the central nervous system was insufficient (12). This was the first in a long line of rapidly changing criteria for MS in attempts to remove the ambiguity of MS diagnosis; however, for some patients it would take years for their MS diagnosis. In 1965, a new addition to the MS criteria was published for achieving a uniform definition to be able to relate findings between clinical therapy trials. The Schumacher criteria included clinical history with a slow or stepwise progression over the course of at least 6 months.
in addition to showing involvement of different regions of the neuraxis. The Schumacher criteria also coined the term “clinically definite MS,” adding to the categories of Allison and Millar, for those patients in which an experienced physician was most certain that the patient had developed MS (13). Still, all of these criteria were based on the patient’s clinical history; no distinct biomarkers were identified. Although it was beneficial for neurologists to have more stringent criteria, it still left the patients waiting several months, or sometimes years for a confirmation of their diagnosis. However, with the invention of two new technologies can more evidence of clinical diagnosis, and laboratory tests of the cerebrospinal fluid for oligoclonal bands or the use of the procedure myelography to identify pathology in the spinal cord were added alongside the typical symptomatic histories to the McAlpine criteria for MS diagnosis (14). As the evolution of MS criteria continued with the Poser criteria, definitions were agreed upon for an MS episode, however, clinical and paraclinical evidence of a lesion and lesions in the gray matter could not be included when establishing the disease diagnosis (15-17). Currently, for the diagnosis of MS as described by the McDonald criteria, a patient’s clinical and paraclinical examinations must show dissemination of demyelinating lesions both in time and space, meaning more than one region of the central nervous system must be affected and each symptom must transpire for at least a 24 hour duration and occur at least 1 month apart from the previous symptoms (18). These criteria were important to exclude alternative diagnoses, such as: neurosyphilis, neuromyelitis optica, Lyme disease, systemic lupus erythematosus, acute disseminated encephalomyelitis, etc. The International Panel on Diagnosis of MS is a cohort of MS specialists who meet regularly to determine and update the criteria in order to promote a higher specificity and sensitivity of the diagnosis of MS worldwide (19). This is of particular importance and will be explained further during the discussions of MS drug treatments.
5.1.2 Evaluating Neurological Disability

It became increasingly important to be able to express and quantify the accumulation of MS patients’ disability over their disease course for comparisons between patient populations in clinical trials, as well as for correlating the amount of disability with the underlying pathology. Therefore, the expanded disability status scale (EDSS) was developed for just this purpose. This ordinal scale is comprised of 19 steps between 0 and 10, with incremental increases of 0.5, representing stepwise increases in disability. A score of 0 would describe the neurologically normal individual, following a neurological examination by a trained neurologist assessing eight functional systems. On the EDSS scale, a score from 0.5-3.5 would be assigned to a patient for whom there is mild dysfunction in 3 or less functional systems. Patients that receive a score between 4.0-5.5 must show a reduced ability to ambulate; therefore, the distance a subject can walk dictates their progression of the EDSS score at this point. Once a patient requires the use of unilateral assistance for ambulation they progress to a score of 6.0, and bilateral assistance results in a score of 6.5 for the patient. Then the range from 7.0 to 9.5 increases with increased immobility and dependence of the patient, such that 8 is wheelchair bound and 9.0 is bed-ridden. Finally, a score of 10 would mean that the patient died due to MS (20). Although this scale provides physicians and researchers an idea of an individual patient’s general disability, there is subjectivity to the neurologists’ scoring since the definitions of the scores are rather vague. Another inherent problem with the EDSS scoring system is the fact that the middle range of the scale is almost entirely based on ambulation; therefore, changes in vision, cognition, arm function, sensory function, and other neurological manifestations are not taken into account.
Hence patients can remain at a particular score for an extended period of time despite both the patient and neurologist observing changes in disability (21). Together these problems can result in an inability to accurately demonstrate treatment effect in clinical trials, and poor correlations with pathology due to its weighty bias on ambulation. Regardless of the problems with the EDSS, it is still the most established and effective measure of disability accumulation in MS patients.

Another scoring system for MS disability is the multiple sclerosis functional composite score (MSFC). This is a 3-part measurement of arm, leg, and cognitive function of the MS patient. There are three tests; the first is the 9-hole peg test, which is used to measure arm and hand dexterity, then the 25 meter walk to measure ambulation capabilities and lastly the Paced Auditory Serial Addition Test (PASAT) is designed to test cognition. Some of the major advantages to the MSFC are that a trained neurologist is not required to give this evaluation as is the case for the EDSS, and that this assessment includes a cognitive test, which is not part of the EDSS. There are some limitations to this scoring system as well, such as not including any quantitative assessment of vision. There are also practice effects associated with both the PASAT and the 9-hole peg test, which can make interpreting the results difficult (272).

5.1.3 Magnetic Resonance Imaging in MS Diagnosis

The MS diagnostic criteria have been significantly revised over the decades, particularly with the invention and utilization of magnetic resonance imaging (MRI), which allows for in
vivo examination of central nervous system tissue and detection of specific pathological changes associated with MS. MRI detects the protons associated with water molecules or elements with unbalanced electrons, by causing them to spin in alignment or anti-alignment with the magnet (314). This imaging technique allows the observer to view the different water environments within the tissue of interest, in this case the brain or spinal cord. In the normal brain there are three different water environments including cerebrospinal fluid-filled cavities, white matter, and gray matter. However, with disease there is a change in the movement of water molecules within the region of pathology. This change can be observed on the MR images as a change in signal where the pathology is occurring. Brain MRI scans are routinely performed on individuals suspected to be suffering from MS, in order to identify abnormalities on the scan that are indicative of MS pathology such as abnormal signal in the white matter, particularly surrounding the ventricles. The use of gadolinium (gad) enhancement administered intravenously prior to an MRI scan allows for the visualization of breaks in the blood-brain barrier, allowing the entrance of blood contents to flow into the central nervous system tissue. The regions where these breaks occur are thought to be acute active sites of inflammatory-mediated demyelination. Lesions that gad-enhance will eventually close the disruption in the blood-brain barrier and no longer enhance, which suggests that these lesions are more chronic. Therefore, identifying these new lesions in addition to lesions which are located in another region of the brain but do not gad-enhance, alongside a typical MS clinical history would be enough for the diagnosis of MS.

MRI can also be performed on spinal cord to identify MS lesions, although this is rarely acquired as compared to brain MRI scans because spinal cord MRI scans are fraught with imaging artifacts due to movement of the chest cavity as a result of the heart beating, breathing and the small diameter of the spinal cord (314). Nevertheless, spinal cord imaging can greatly
assist in the diagnosis of MS, particularly in patients with brain MRI scans that are negative for MS-related lesions. This is most commonly the case for patients with a primary progressive disease course and is discussed later in this chapter. Gadolinium enhancement is not commonly observed within the spinal cord, making it more difficult to determine when these lesions developed and making it more challenging to understand what role inflammation plays in their pathogenesis. MRI can not only expedite the diagnosis of MS, but it also allows for the monitoring of the disease over its duration and at times aids in the differentiation between MS variants.

5.1.4 Clinical Manifestations

The spectrum of MS encompasses a few variants which are differentiated based upon disease progression, pathology, and/or radiological findings. The most common clinical manifestation of the disease, afflicting roughly 80% of the MS population, is known as relapse-remitting MS (RRMS). This form of MS affects females two to three times more frequently than males. The onset of the disease is usually in the second or third decade of life. RRMS patients rarely die in this condition; rather, they transform into secondary progressive MS (SPMS) patients, where the lifespan is decreased. This RRMS stage of the disease is marked by exacerbations of neurological deficits with highly unpredictable levels of recovery following the episode (Fig. 1). Patients with this form of the disease vary greatly in their symptoms, as well as in the time between each relapse. The number of lesions identified by MRI does not always correlate with symptoms, meaning that some lesions are not in articulate portions of the brain.
and therefore, do not correspond with an observable neurological disability. Thus a patient’s first attack or episode may not be from the first MS lesion that developed. The disability that accompanies demyelinating lesions is thought to occur when the myelin sheath produced by oligodendrocytes is removed from around axons. Edema and conduction block at nodes of Ranvier are thought to cause rapid onset disability, followed by demyelination. Current MS therapies are aimed at reducing the number of these episodes by inhibiting the peripheral immune system and/or their entry into the central nervous system. There are three ways in which axonal conduction (saltatory conduction requires myelin and nodes) can be restored within demyelinated lesions: 1) resolution of the edema; 2) spontaneous redistribution of sodium channels along the entire demyelinated axolemma such that non-saltatory conduction can occur; and 3) during early stages of the disease process, demyelinated axons can be spontaneously remyelinated by newly formed oligodendrocytes. When these events occur, nerve transmission is restored and the prior disability resolves to a varying degree, depending on the depth of the repair mechanisms involved. Unfortunately, not every relapse comes with complete remission of the lost function and disability eventually begins to accumulate. Despite the immunomodulating treatments for the RRMS stage of the disease, the disease still advances and eventually most of these patients will proceed to SPMS. At this point, the neurological deterioration becomes permanent and continuous over time, while all treatments that were effective in RRMS no longer show any benefits in SPMS. The accumulation of new MRI lesions decreases during this stage of the disease, but the progression of disability results from the loss of axons and neurons from within the demyelinating lesions. As more of the axons are lost, the brain’s ability to compensate for these losses decreases and disability accumulates. During the progressive stages
of MS, neuroprotection may need to become the main focus of drug treatment to prevent the continuous neurological deterioration.

**Schematic of Multiple Sclerosis Progression**

![Schematic of Multiple Sclerosis Progression](image)

Figure 1. Schematic of the disease progression of multiple sclerosis patients. The red line is the clinical threshold at which symptoms are evident upon neurological examination. The black line represents the RRMS and SPMS disease course, with relapses that develop and recover to varying degree and then eventually a steady continuous neurological decline. The gray line shows the primary progressive continuous decline from onset. The arrows at the bottom mark the detection of new MRI white matter lesions. The blue line shows brain volume loss, which begins early in the disease and continues throughout the disease course.

Primary progressive MS (PPMS) is the clinical manifestation of MS in roughly 10-15% of the MS population, with a gradual and continuous worsening of neurological disability from disease onset (Fig. 1). The onset of this subtype is usually later in life (50 years or older) than
RRMS patients, but due to the rate of progression of the disease, these patients hit disability milestones at the same times as RRMS patients, typically reaching an EDSS of 6.0, six years after developing the disease (22). Interestingly, there is no difference in incidence between males and females to develop PPMS. Relapses do not occur in the PPMS patients; however, plateaus in disability may appear without any recovery. The most common initiating symptom for this type of MS is progressive paraparesis, although progressive visual loss, progressive hemiplegia or progressive cerebellar/brainstem disturbance are also possible but less frequent initiating symptoms (22). Interestingly, PPMS patients have been shown to have better psychological functioning than RRMS patients (273). This is thought to be possibly due to the fact that PPMS patients do not have to cope with the spontaneous and unexpected relapses, or because of the decreased number of abnormalities on their cerebral MRIs compared to RRMS patients. Another possibility is the duration of suffering with the disease is not as long for PPMS patients, since they develop the disease later in life. In other words, the longer that an individual has to deal with this long term illness the worse their psychological functioning becomes. Similar to SPMS patients, PPMS patients do not respond to the current disease modifying therapies utilized for RRMS.

Progressive-Relapsing MS (PRMS) affects less than 5% of MS patients. PRMS begins with a progressive decline and is punctuated by intermittent episodes without remission of the symptom, along with continuous decline in between each of the relapses (23). The onset for this form of MS is about 40 years of age or later (24). The relapses, however, can commence as late as 10 years into the progressive disease making this form difficult to diagnosis in some patients and very unpredictable (24, 25). Relapse rates for these patients is typically less than 1 relapse per year, but 38% of these patients have 4 or more relapses a year (24). Progressive-Relapsing
MS affects both women and men, but the incidence is slightly higher in women (1.4-2:1) (25, 26). Little is known about this group of MS patients regarding the effectiveness of disease-modifying therapies, however the antineoplastic drug, mitoxantrone appears to be effective at reducing the number of relapses and disability progression in some PRMS patients (27).

Marburg’s MS is one of the most severe and progressive forms of MS, with these patients usually dying within months to a year after disease onset. As described in a review by Herndon, the description of Marburg’s MS came from Otto Marburg in 1906 (28, 36). This variant commences with a rapid and continuous progression of demyelination typically in either the cerebrum or brainstem. The MRIs of these patients usually consists of multiple confluent lesions that increase in size. The very few patients that do survive past the first few months will continue with a RRMS-like disease course for the remainder of their life. These demyelinating lesions are completely devoid of myelin, which can be observed in the many T cells and macrophages within the lesions, and with a limited number of lesions showing evidence of remyelination. The lesions are described as being more destructive pathologically than typical MS lesion (36). Due to the rarity of the disease and the rapid disease course, no clinical trials for efficacy of disease modifying therapies have been performed specifically in this MS variant.

Initially named leuko-encephalitis periaxialis concentrica, Balo’s Concentric Sclerosis like Marburg’s variant, is often thought of as a severe and rapidly terminal form of MS. This rare variant is mostly monophasic due to the swift progression and fatal outcome within weeks to months of clinical onset. However, a very small subset of these patients recovers and progresses with a RRMS disease course. Typically, this form affects young adults (average age of 34 years) with clinical manifestations including headache, aphasia, cognitive and/or behavioral changes, and seizures. Similar to RRMS, there is a ratio of 2:1 for females to males developing this
disease variant. Pathologically, white matter lesions demonstrate an unusual pattern of demyelination in which the core of the lesion is demyelinated, but alternating rings of demyelination and preserved myelin or remyelination radiate outward. Although these lesions can be quite large, they do not affect the cerebral gray matter, but have been shown to affect the cerebellum, brainstem, spinal cord, and even the optic chiasm. These discrete lesions with concentric bands of demyelination are visible on MRI acutely and therefore can be diagnosed pre-mortem; however, within a few weeks these lesions no longer enhance with a gadolinium ring at the peripheral edge of the lesion. Initially these lesions have a hypointensity in the center on a T1-weighted scan with alternating rings of isointensity and hypointensity. On T2-weighted scans these lesions can form different patterns which have been described as: a storm-center with hyperintense lamellae around the periphery, or mosaic, rosette, or carnation patterns. It is not yet known whether these bands of demyelination occur simultaneously or form sequentially; identification of this pattern of demyelination may lead to a better understanding of the pathogenesis of these particular lesions (28-31).

One common mistaken differential diagnosis for Balo’s lesions is tumefactive demyelination, since both lesions can be quite large in size and Balo’s lesions can evolve to look much like tumefactive lesions. Tumefactive MS lesions are 2cm or greater and are often also mistaken for brain tumors, biopsy results show a demyelinating lesion. Typically, these lesions are supratentorial and therefore result in multifocal symptoms, affecting motor, cognitive, and sensory functions and they occasionally produce seizures (32, 33). Although the course begins with large space occupying lesion, clinically these patients will progress with an RRMS-like disease course, with perturbations and remissions. The formation of the large space-occupying lesions occurs as the presenting pathology for 61% of the MS patients that will develop
Tumefactive MS (33). On occasion the large space occupying lesions form as a remission in the disease course, instead of the initiating event. The frequency of MS patients with tumefactive MS is estimated to be approximately 1 in 1000 MS patients; therefore, studies of this population are scarce and often retrospective, and no sex differences have been observed for this MS subtype (34). The size of the lesion does not correlate with age of onset, age at lesion biopsy, gender, or clinical course, but had a weak correlation \( r = 0.25 \) with the EDSS at follow up, such that those with a lesion greater than 5cm had a higher mean EDSS than those with lesions less than 5cm in size (33). These lesions will frequently show a mass effect and edema by MRI (35, 36), as well as an open ring enhancement following administration of gadolinium, although other patterns of enhancement have been described (33). Ultimately, it is unknown what mechanisms result in these larger cerebral lesions in some patients and smaller to no lesions in others.

Benign MS is a unique disease course in which the disease progresses very little and disability rarely accumulates in these patients over decades following the first manifestation of the disease. The definition of Benign MS has changed over the years but currently refers to when a patient has minimal to no disability (EDSS of \( \leq 3.0 \)) 10 or more years from the onset of the disease. These patients are estimated to be about 7% of the MS population with no differences between the sexes (37), but due to the differences in definition of these patients, the exact prevalence is unclear (38). Usually the disease course is monosymptomatic, is associated with optic neuritis, and has onset before 40 years of age, an absence of pyramidal involvement, very few relapses, and minimal neurological impairment in the first 5 years (39). Interestingly, among patients which presented with fewer than 10 brain lesions by MRI, roughly 75% will have benign MS (37, 40), which show a preservation of both concentration of N-acetyl aspartate and magnetization transfer ratio (41). Disability in MS patients is strongly weighed on motor and
ambulatory dysfunction, but cognition can also be greatly affected. In a study of 163 patients with benign MS, 45% were deemed cognitively impaired after failing 3 or more neuropsychological tests, suggesting that cognitive impairments can progress in the absence the motor dysfunction (39). Disease modifying therapies are not commonly administered to these patients because of the belief that it is probably not necessary and may not be beneficial to their disease course.

Pediatric MS is defined as an MS onset under the age of 18 years while fulfilling the McDonald criteria for MS diagnosis, which is estimated to be less than 5% of the MS population (1) with roughly 8,000 – 10,000 children affected in the United States. The diagnosis is more challenging in children due to the frequency of other childhood disorders with similar symptoms and characteristics such as Acute Disseminate Encephalomyelitis (ADEM) (42); therefore, these other conditions must first be ruled out as possible alternative diagnoses. Demyelinating lesions are evident by MRI in the brain and/or spinal cord, similar to those observed in adult MS subtypes (43). The symptoms are similar between children and adults, such as optic neuritis, hemiparesis, ataxia or spinal cord symptoms; however, children are more likely to have seizures and mental status changes than adults. This is likely due to the fact that the brain still developing and connections are still forming. Currently, there are no approved disease-modifying therapies specifically for children, but some treatments for adults have been tried on children, with greater variation in the efficacy.

The MS spectrum varies greatly from the slowly progressing to the rapid and severe disease course; however, it is unknown what pathogenic mechanisms gives rise to the different MS clinical manifestations or what factors contribute to the risk of developing the disease. It is possible that the pathogenic mechanism is generally the same in all of the MS variants, but
genetic and environmental factors may play a role in the specific subtype of MS that the individual develops. Although each of the subtypes is unique, the overwhelming common thread among all these MS variants is the demyelination that occurs throughout the neuraxis, particularly within the cerebral white matter. Examination of the pathology present within the white matter of MS subtypes gives us insights into the possible mechanisms of demyelination occurring within these patients.

5.1.5 MS Pathology

The pathology relating to MS can be observed throughout the central nervous system, however the white matter demyelinating lesions are the pathological hallmark of this disease and must disseminate in time and space before the diagnosis of MS is pronounced. The white matter pathology for all subtypes of MS has been studied for centuries and what we presently know is that the most common regions of the brain to be affected by demyelination are the periventricular white matter, the corpus callosum, the juxtacortical white matter, the spinal cord, optic nerve, and the brainstem (44). However, as Brownell and Hughes (45) stated, “no region is immune from the occurrence of plaques,” within the central nervous system. As mentioned before lesions can be visualized on MRI scans as well as seen macroscopically in postmortem tissue by the discoloration of the tissue in the area of the lesion due to the loss of the iridescent myelin which gives the tissue its white coloring (46). Through the postmortem histological analysis of white matter lesions a chronological classification was established based upon myelin integrity and the activated inflammatory infiltrating cells (46); therefore by examining the microglial activation
through immunohistochemical techniques, the “age” of the white matter lesion can be estimated. White matter lesions commence in the acute active stage with breakdown of the blood-brain barrier, extensive infiltration of peripheral inflammatory cells (mostly monocytes to phagocytize the myelin), demyelination of the axons, activation of the nearby microglia (the resident immune cells of the central nervous system), astrogliosis and axonal injury (44). The breakdown of the blood-brain barrier can be visualized by immunohistochemical stains for serum proteins such as IgG, albumin or fibrinogen (47). These acute lesions can be differentiated from chronic lesions by MRI scans following the infusion of gadolinium-DTPA (discussed in greater detail later in this chapter). The gadolinium enhancing lesions are most often observed in RRMS; however they are occasionally present in the SPMS stage (21). After a few months, the acute active white matter lesions will eventually develop into a chronic active lesion where activated microglia and macrophages are present at the borders of the lesion following the evacuation of the phagocytes from the interior of the white matter lesions. While the microglia and macrophages are continuing to remove myelin, axonal transection and neuronal loss also continue to transpire as described by Trapp and colleagues (48) due to the damaging environment within the white matter lesion core. Chronic active lesions can be seen on T2-Weighted MRI scans as hyperintensities (bright spots) within the cerebral white matter which delineates where pathology is present (21). Finally, after several years, white matter lesions advance to chronic inactive lesions, where only activated microglia are present at the border of the lesion with continued ongoing neuronal and axonal damage. These lesions have been in the brain the longest and no longer have inflammatory infiltration causing breakdown of the blood-brain barrier. Furthermore, these chronic inactive lesions appear on T1-weighted MRI scans as hypointensities (dark spots), also referred to as “black holes” within the cerebral white matter (discussed further
later in this chapter). Additional pathological observations seen in white matter lesions are reactive astrogliosis, oligodendrocytes loss, sustained and extensive inflammation within perivascular cuffs, and axonal swellings and degeneration (49). The irreversible neurological disabilities observed in this demyelinating disease are thought by all, to be caused by axonal damage and loss in and around the lesions. Trapp and colleagues (48) demonstrated that active white matter lesions from MS patients with disease durations ranging from 2 weeks to 27 years contained approximately 11,000 axonal terminal ends per mm$^3$. Demyelinated axons are vulnerable to the milieu of neurotoxic factors with in the microenvironment of demyelinated lesions, resulting in axonal transection and ultimately degeneration. The axonal degeneration during the acute phase of lesion development is likely caused by infiltrating inflammatory cells, not from a direct attack on the axons themselves but rather from the release of matrix metalloproteases, cytokines, free radicals, etc. These molecules damage the axon as well as mitochondria within the axon, leading to an energy imbalance similar to that seen during ischemic events (315). Similarly, chronic lesion borders contained over 3,000 terminal ends per mm$^3$, as compared to control tissue, which was comprised of < 1 transected axon per mm$^3$ (48).

There are several possible mechanisms for this pathology. Inflammatory secretions during the acute phase could have damaged the axon and now the chronically demyelinated axon having lost the myelin-derived support, shows disruption of axonal ion concentrations resulting in intracellular Ca$^{+2}$ accumulation (50). In an attempt to restore conduction, axons redistribute sodium channels along the bare portion of the axon. This process causes an increased influx of Na$^+$ and a higher energy demand on the Na$^+$/K$^+$ ATPase to remove the intracellular Na$^+$. Eventually, the increased Na$^+$ levels will cause a reversal of the Na$^+$/Ca$^{+2}$ exchanger, and the subsequent accumulation of Ca$^{+2}$ activates proteases, the release of more Ca$^{+2}$ from the
ryanodine receptors on the endoplasmic reticulum, impairment of axonal transport, and other toxic effects. Axons may not succumb to degeneration if the bare axon is remyelinated by a newly matured oligodendrocyte. In this case, reorganization of the underlying microtubules, redirection of Na+ channels to the nodes, and restored trophic support will help repair the axon and increase survival. Even with remyelination, axons are still vulnerable to persistent disease conditions, which may lead to the axon being demyelinated again before transecting and degenerating. Additionally, remyelination capacity is limited, since very few white matter lesions completely remyelinate into shadow plaques, but rather most only show remyelination at the border of the lesion. The white matter is not the only location of demyelination and damage; other central nervous system regions with MS related pathology may also contribute to the neurological symptoms associated with MS.

Although white matter lesions are a major focus of MS research, gray matter demyelination is now recognized as being a prominent pathological feature in postmortem MS brains. Incidentally, the gray matter pathology had been mostly ignored in the past, likely as a result of it not being identifiable macroscopically or by conventional MRI techniques. Gray matter demyelinating lesions do not show a color change with the loss of myelin, in contrast to white matter lesions, due to the fact that the gray matter has less myelin present and, hence the loss of myelin in the gray matter is less noticeable macroscopically. Subsequently, the extent to which gray matter was affected in MS patients went largely unnoticed and was therefore underresearched until recently. Through histopathological studies gray matter lesions have been found in the cortex, basal ganglia, hippocampus, thalamus, the brainstem and spinal cord (45, 47, 49, 51-56). Brownell and Hughes (45) showed that 26% of hemispheric lesions were outside of the white matter, with 17% along the leukocortical junction, 4% in deep gray matter regions, and 5%
were cortical lesions. Interestingly, gray matter lesions are not visualized on conventional MRI
scans, in part due to the absence of inflammatory phagocytic macrophages and T-lymphocytes
and due to varying degrees of edema unlike their white matter lesions counterparts. Also, there
are MRI analysis difficulties associated with partial volume effects at the border of the brain
surface and cerebrospinal fluid (47, 51, 52). Partial volume effects are where a single voxel in
the image contains both cerebrospinal fluid and brain tissue, making it difficult to interrupt the
low signal within that voxel. The low signal could be because of the cerebrospinal fluid or due to
demyelination. Therefore, gray matter lesions must be identified through immunohistochemical
analyses for quantification of extent and frequency as well as investigation of other types of
pathologies besides demyelination.

Cortical lesions have been shown to differ from cerebral white matter lesions in a few
ways, the first being the absence of peripheral blood-borne monocytes and lymphocytes in or
around the lesions (51), with the exception of the leukocortical lesions, as well as in an intact
blood-brain barrier (57). Unlike white matter lesions, subpial and intracortical gray matter
lesions cannot be chronologically classified, because of the lack of infiltrating immune cells. It
is difficult to determine whether demyelination is active or not with only the presence of
activated microglia within these gray matter lesions. Nevertheless, we have described a pattern
for classification of cortical lesions by their location and extent of demyelination within the
cortex, with type I being leukocortical lesions in which the demyelination crosses through the
gray/white matter border; type II intracortical lesions are usually small areas of demyelination
found strictly within the cortex and surround a blood vessel; and type III are subpial lesions that
span from the pial surface and continuing down into deeper layers of the cortex, usually stopping
at layers 3-4 but, occasionally including all 6 cortical layers without disturbing the underlying
white matter. Subpial lesions can extend across several gyri (29, 45, 51). When the cortical portion of leukocortical lesions were compared to the white matter portions there was a thirteen fold reduction in the amount of CD68-positive microglia/macrophages and a six fold reduction in the amount of CD3-positive T-cells in cortical portion of these lesions (51). Type III or subpial lesions are the most abundant type of cortical lesion and likely contribute the most to the patient’s neurological dysfunction (47, 52). Therefore, they require further investigation of the underlying mechanism of demyelination. Secondly, gray matter lesions resulting from the loss of oligodendrocytes, neurons and their axons and synapses (47) have a better propensity to be remyelinated than white matter lesions (58). This difference in capacity of remyelination is important and may lead to new therapeutics targeting activators of remyelination or removal of remyelination inhibitors.

Several studies have estimated the distribution of cortical lesion load, which may be equal to or possibly even exceed that of the cerebral white matter lesion load (29, 45, 52). One study demonstrated cortical lesion frequency by examining four predetermined cortical regions from a select number of classical postmortem MS brains. More than 25% of the cortex was demyelinated in these cases, which was surprisingly higher than the 6% of demyelinated cerebral white matter (45). The cingulate cortex had the highest prevalence of demyelination with a mean percent demyelinated area of 43.8%, while the temporal cortex had 27.7% demyelinated, followed by the frontal cortex with 24.1% and the parietal cortex with 11.5% demyelinated. Several histopathological studies have demonstrated the specific vulnerability of the cingulate gyrus for cortical subpial lesions as well as atrophy (199; 236; 274); however, it is unclear what leads to the specific vulnerability of this region or any region of the cortex to demyelination. Although in the case of the cingulate gyrus it is surrounded by deep sulci, which could leave it
vulnerable to factors circulating within the CSF. Subpial lesions contain transected neurites, apoptotic neurons and decreased neuronal, glial and synaptic densities (51, 59, 60). The loss of neurons and their axons is not only in the regions of demyelination, normal appearing gray matter was also shown to have a loss of 33% of axons and a reduction of 13.6% in neuronal size compared to cortex from control tissue (61). These synaptic, neuronal, glial and axonal changes in the gray matter, regardless of myelination status, is evidence of a neurodegenerative process occurring in MS patients. However, it is poorly understood how these mechanisms of demyelination and degeneration interact to form MS cortical pathology. It is also unknown how these extensive cortical lesions develop. It is not clear whether the mechanism of subpial cortical demyelination is similar to or different from the process active in cerebral white matter lesions.

One current theory regarding the formation of cortical lesions in the field is that B-cell follicles located in the perivascular space of the cortical vessels of the leptomeninges, particularly those in deep sulci of the cortex, release a molecule into the CSF or surrounding parenchyma (possibly a cytokine or a chemokine), which penetrates into the surface of the cortex and in some manner causes the subpial demyelination (62, 63). A small collection of studies have identified inflammation of the leptomeninges of post-mortem tissue, however others detected no association of this inflammation with the subpial lesions at all (62-66). The axonal injury within subpial lesions has been shown to be accompanied by extensive microglial activation and sporadic inflammatory infiltration in the brain and meninges (64). This is one possible mechanism for the formation of cortical lesions, but not all reports confirm these findings. Alongside demyelination, oligodendrocyte cell death occurs and may be the primary cause of demyelination, since they produce the myelin sheath within the central nervous system (62-66). It is possible that the infiltrating factors released by B-cells from the inflammatory
aggregates within the follicular structures of the meninges could be directly toxic to oligodendrocytes or the myelin sheath resulting in oligodendrocyte death. It is also possible that these inflammatory factors may be acting indirectly through activation of the microglia in the brain parenchyma, which in turn could be playing a role in the mechanism of demyelination of the cortex. Microglia are innate immune cells in the central nervous system (67-69), and have been shown to phagocytize debris (70, 71), as well as playing a role in repair mechanisms (67, 72). Once activated by these infiltrating molecules, microglia could destroy myelin and oligodendrocytes (70, 71). However, all of these proposed mechanisms of oligodendrocyte cell death have yet to be proven and therefore it is still unknown. Potential factors released by the B cell follicles are IgG or complement molecules, however, neither IgG nor complement has been detected within subpial lesions (73, 74). Oligodendrocytes could be killed due to a metabolic insult resulting from reactive nitrogen or oxygen species affecting the mitochondria, released by the inflammatory cells. Oligodendrocytes can maintain up to 50 myelin internodes (75), and the maintenance of all the myelin lipids and proteins, as well as the trophic support that they provide the neurons, leaves these cells vulnerable to metabolic dysregulation inflicted by immune effectors, possibly leading to cell death. These potentially detrimental hematogenous cellular and molecular factors could result in the demyelination of the cortical gray matter. Exploration of postmortem tissue for these factors may lead to targeted therapies for preventing demyelination.

The hippocampus has been of particular interest to MS researchers in part due to its functional role in learning and memory, especially episodic and anterograde memory functioning, which are the most frequently impaired cognitive modalities in MS. This is significant to the MS population because 40-60% of MS patients present with cognitive deficits
over the disease course (76). Furthermore, it has been demonstrated that the hippocampus is especially sensitive to damage from multiple insults, for example repeated influx of inflammatory cells (77, 78). It is estimate that between 53-79% of MS hippocampi are demyelinated (56, 77,79). Extensive demyelination in the hippocampus has been demonstrated in several studies of MS patients, with one reporting an average of 30.4% demyelination of the hippocampus and another estimating an average of 46.6mm² of demyelination (56, 77). These demyelinating hippocampal lesions were found most often to be chronic lesions affecting both gray and white matter and to be located along the subpial or subependymal surfaces of the hippocampus (77). Decreases in the number of neurons have been demonstrated for several of the regions in the hippocampus as great as 27% of neurons in the CA1 and 29.7% in the CA2-3. Moreover, a reduction of 22.3% in the overall cross-sectional area of the hippocampus in MS patients as compared to controls was also observed (77). Volume loss was observed by MRI to be selectively lost in the CA1 region of the hippocampus in RRMS by an average of 12%, with 25% volume loss in SPMS patients. This hippocampal atrophy also affected other regions of the hippocampus with a 13% reduction when compared to controls. These changes in total hippocampal volume and regional volume losses had a moderate correlation with a worsening of performance on cognitive tasks such as word-list learning and memory encoding tasks (78). In addition, molecular changes in demyelinated hippocampi of MS patients compared to myelinated hippocampi from MS patients showed decreases in neuronal proteins involved in axonal transport, synaptic plasticity, glutamate neurotransmission, glutamate homeostasis and memory/learning (79). These alterations as a result of demyelination in the hippocampus, are likely to be causing the memory dysfunction associated with MS (79), which can greatly impact these patients’ quality of life and abilities to function on a daily basis.
The thalamus, a deep gray matter structure of the cerebrum, is also affected by MS pathology. The thalamus is located at the very center of the brain and abuts the lateral ventricles. It is a unique structure because of its many reciprocal connections within the subcortical white matter connecting spinal cord projections and the cerebral cortex. These connections are very important to its function as the relay structure of the brain, but may leave the thalamus vulnerable to Wallerian degeneration due to axonal transections of its connections from a location where an MS demyelinating lesion has developed. Cifelli and coworkers (55), estimated that in the thalamus there was a 35% total reduction in neuronal numbers in MS patients as compared to controls. Furthermore, thalamic atrophy has been identified as one of the first MRI detectable changes in MS patients (80). Thalamic lesions have been reported but seem to be a rather rare occurrence, but when present 87.5% were purely gray matter lesions and the rest were mixed gray matter/white matter lesions (81). Thalamic demyelination appears in both clinically isolated syndrome and RRMS patients along with demyelination in the caudate nucleus, hypothalamus, globus pallidus, and putamen, suggesting that this early deep gray matter demyelination is independent of the white matter lesions (82).

Spinal cord pathology in MS can develop independently of the changes arising in the brain. Lesions in the spinal cord typical invade the gray and white matter, not respecting the border between these two tissue regions, much like leukocortical lesions in the brain (81, 83). Demyelination, active inflammation, severe gliosis, and axonal damage have all been observed within spinal cord lesions (54, 83). For all MS subtypes the proportion of gray matter that was demyelinated in the spinal cord (roughly 33%) was found to be significantly greater than the proportion of demyelinated white matter (20%) (83). Although the gray matter demyelination can be extensive, no difference has been found in the amount of gray matter demyelination
between the different levels of the cord (83). However, the white matter demyelination was
greatest in the cervical level of the spinal cord (83). When the corticospinal and sensory tracts of
the spinal cord were examined from 55 postmortem MS patients, it was revealed that the spinal
cord area and axonal densities were reduced at all levels of the MS spinal cord in the
corticospinal tract compared to controls, but only in the upper regions of the MS spinal cord for
the sensory tracts were significantly reduced compared to controls (84). The axons that were
preferentially lost seemed to be the small caliber fibers (less than 3 um2) for both the sensory
and corticospinal tracts (84). Regardless of the fiber tract, an average of 68% of axons were
shown to be lost within spinal cord lesions from progressive MS patients with a EDSS score of
7.5 or higher (54). Spinal cord chronic demyelination and axonal loss are major contributors of
the irreversible neurological disability in MS patients.

5.1.6 Magnetic Resonance Imaging Metrics Related to MS Pathology

Magnetic resonance imaging (MRI) allows for the viewing of internal human anatomy in vivo by using a large magnet (measure of the strength in tesla, T) that aligns all of the hydrogen protons with the magnetic field of the intended target. A brief radio-frequency pulse is applied to excite the hydrogen nuclei to a higher energy, allowing them to spin in an anti-parallel fashion to the magnetic field. This magnetization or higher energy state will relax over time, and different tissues relax at different rates depending on their make-up of water and/or complex macromolecules, which in turn will affect the contrast of the resulting image. The relaxation of the excited hydrogen nuclei is what is detected at the time of imaging, so as regions that have
fast relaxation such as fats or myelin will appear very different on the image to water or cerebrospinal fluid, which have slower relaxation times. Therefore, a change in the microenvironment, like those observed in pathological states, changes the relaxation state to the surrounding tissue and hence the signal detected by the MRI and results in an abnormal region on the image. There are two types of conventional MRI modalities, the first is T2-weighted scans which measures T2 relaxation. This is the point at which 63% of the transverse magnetization has decayed after the radio-frequency pulse. The second type is T1-weighted scans which, similar to T2, measures the relaxation state when 63% of the longitudinal magnetization recovers and is called T1 relaxation (314). MRI has been a major tool for diagnosis and understanding the progression of pathology during the MS disease course. Imaging individuals with MS at various times during their disease course allows for the examination of how pathology changes over time, in addition to the differences in underlying pathology between abnormal signal on a T2-weighted image from those on a T1-weighted image. One particular MS pathology detected by both T1 and T2 MRI is the hallmark cerebral white matter lesions. These lesions appear both acutely and chronically throughout the neuraxis and often increase in number over the disease duration, but develop far more commonly during the RRMS phase of the disease compared to the progressive disease stages.

Many studies have focused on attempting to understand the relationship between T2 and T1 lesions as well as their relationship with the underlying pathology and neurological disability. T2-weighted MRI white matter lesions can be substantial in number and volume by the time of the initial clinical symptoms, which likely reflects the subclinical or “silent” events that occurred in non-functionally sensitive areas of the central nervous system. This fact demonstrates that not all lesions will result in a neurological disability, and therefore the correlation between disability
and number of lesions is not likely to be strong. Furthermore, it indicates that disease onset may occur long before clinical manifestation begins. The disability scores of patients based on the EDSS scale correlates relatively poorly with the T2 brain white matter lesion volume (r=0.2). There are many factors which contribute to this modest correlation, such as location of the lesion, the redundancy of particular neural pathways, functional reorganization in response to tissue injury, lack of pathological specificity of T2 lesions, and the contribution of subtle changes in normal appearing gray and white matter, which are not included in the T2 lesion load (85).

Cerebral white matter lesion volume measured on T2-weighted MRI scans is thought to be a representative measure of the total amount of pathological tissue, including demyelination, while the counts of these lesions represents the disease activity over an interval of time (21), meaning that patients with several white matter lesions by T2 MRI are more likely to have a highly active form of MS as compared to those that have very few to none. The T2 lesion volumes have been shown to increase the most during the RRMS phase increasing by 5-15% annually (86, 87), but interestingly, the lesion volumes only increase by 3.6 to 9% annually in the SPMS phase (88, 89). Initially these T2 lesions expand in size for a period of 2 to 8 weeks before they reach their maximal size, after which they will then shrink over the following weeks to the essential core of the lesion, or T2 footprint. The change from expanding to shrinking is likely due to repair of the blood-brain barrier, which prevents the further infiltration of peripheral immune cells and reduces the demyelination activity and the edema that eventually subsides shrinking the lesion back to its demyelinating core, in addition to the loss of oligodendrocytes and some axonal loss within the lesion. This stabilized footprint of the T2 MRI lesions generally does not change in volume beyond this point; however, on rare occasions, individual lesions can expand at chronic time points, and this is thought to be a result of a reactivation of the peripheral immune system.
and disturbance of the blood-brain barrier, likely producing more accumulation of pathology and possibly a loss of the remyelination capacity (90, 91). Ultimately, the T2 cerebral lesion load does not portray the severity of each lesion or the overall damage occurring within the patient and therefore is limited in determining disease progression, particularly since in the progressive stages new T2 lesions are rare but disability continues to accumulate.

Distinguishing newly forming and chronic lesions can aid in the diagnosis of MS by demonstrating dissemination of lesions over time. This is accomplished by the use of gadolinium (gad) enhancement, which identifies acute breakdown of the blood-brain barrier and acute lesions. Therefore, the presence of a gad-enhancing T2 positive lesion and T2 only positive lesions provides MRI evidence for dissemination of lesion formation. This expedites the diagnosis of MS. Gadolinium is a contrast agent used to visualize breakdown in the blood-brain/spinal cord barriers (BBB). When the BBB is intact, the gadolinium remains strictly within the blood vessels; however, when there is a breach in the BBB the gadolinium enters the central nervous system tissue at the location of the leak, resulting in an enhancement of the surrounding lesional area. White matter lesions that enhance with gadolinium can occur at any stage of the disease; however, their frequency decreases with increase of age and duration of the disease, as well as decreases in relapsing rates. When MS patients had monthly MRI scans performed, 50% of the clinically isolated syndrome patients contained at least one gad-enhancing lesion (92); 50-65% of RRMS (87, 93, 94), 35-50% of SPMS (95-97) and 14% of PPMS had gad-enhancing lesions (95, 98). The number of gadolinium enhancing lesions correlates with relapses in the following 6 months for RRMS patients (299) and a higher number of gadolinium enhancing lesions correlated with a more rapid deterioration over the subsequent 5 years in SPMS (300). It has also been shown that the number of gadolinium enhancing lesions is significantly higher in
the RRMS patients than in SPMS patients (301). This further demonstrates that active inflammatory mechanisms are present more so in the early stages of the disease course, with a higher percentage of patients containing an enhancing lesion in the CIS and RRMS stages than SPMS and PPMS. It also confirms that in PPMS, there is a lesser degree of active inflammation than in RRMS patients. Furthermore, gad-enhancing lesions weakly correlates with clinical relapse rate, but can predict a patient’s number of subsequent enlarging T2 lesions, T2 lesion volume, and T1 black hole volumes (99-103). There is also a weak correlation between the number of enhancing lesions and brain atrophy in the short term, which increases slightly with long term intervals (99, 104, 105), suggesting that more gad-enhancing lesions indicates more inflammatory damage, which can result in axonal damage and loss, and therefore eventual brain tissue volume loss. Gadolinium enhancement therefore is another tool, in addition to conventional MRI metrics, used to assess the progression and level of active processes occurring in the individual MS patients and across MS subtypes.

T1-Weighted MRI scans also detect MS white matter lesions, but differ from T2-weighted images in that these regions appear dark or hypointense and are therefore referred to as T1 black holes. These T1 black holes have been shown to be present in all stages of the disease, with 50% of CIS patients containing at least 1 T1 black hole by MRI (106), which supports that the disease has long been active prior to clinically observable symptoms appearing. All T1-weighted lesions are also detectable on the T2-weighted images, but not all T2 lesions will progress to the level of pathology to result in a T1 black hole. For this reason, T1 black holes are considered to be a result of severe and irreversible pathology (21). This is demonstrated by the observation that approximately 5-20% of T2 lesions are also T1-positive in RRMS and SPMS patients. Histopathological studies have determined that within these T1 black holes, there is
reduced axonal density, myelin loss, increased gliosis, and alterations in the BBB. T2 lesions also have reduced magnetic transfer ratios and reduced N-acetyl aspartate (NAA) levels, which both reflect axonal loss (107). Larger white matter lesions (greater than 2 cm in diameter) are more likely to develop into T1 black holes. Lesions on MRI with longer durations of enhancement, or lesions with ring-enhancement also have a greater chance of developing into T1 black holes (108, 109). Interestingly, MS patients with the apolipoprotein E-ε4 (APOE4) allele have a greater T1 lesion load, raising the possibility that demyelinated axonal degeneration is greater in these individuals (110), which may suggest similar processes of axonal damage between Alzheimer’s disease and MS. T1 black hole lesion volumes have been shown, like T2 lesion volumes, to increase with the disease duration; however, there is at best a weak correlation between these chronic T1-hypointense lesions and neurological disability (99, 111). Although MRI T1 lesion loads are informative about where the chronic and more severe pathology has occurred, they do not relay specific information about the integrity of the structures within the region of abnormal tissue.

One of the major limitations of conventional MRI techniques is the lack of specificity for the underlying heterogeneous pathology (112). Many mechanisms including edema, inflammation, demyelination, remyelination, and gliosis as well as neuronal and axonal loss can contribute to and result in a similar appearance of hyperintensity on T2 weighted images (113). Magnetic transfer ratio (MTR) is a measurement of proton spin magnetization exchange between molecules particularly those which are larger proteins or molecules such as myelin, and this exchange process reflects the overall characteristic of those macromolecules within the environment (21). Therefore, when a particular region has a high MTR level this indicates that the capacity of the molecules within the brain tissue have a high exchange of the magnetization
with the surrounding water molecules. However, a low MTR designates a reduced exchange of the magnetization with the water molecules. MS white matter lesions typically have low MTR values which suggests a decrease in the integrity of macromolecules within the brain tissue matrix caused by demyelination, edema and/or axonal swelling (114, 115). The MTR values of normal appearing white matter are significantly higher than in T2-weighted white matter MRI lesions, suggesting that demyelination likely occurred in regions with MTR decreases (116). MTR values that show marked decreases over time are likely to correspond to severe tissue damage, since strong correlations have been demonstrated between MTR and the percentage of preserved axons and demyelination (114). MTR changes have also been associated with the duration of lesion gad-enhancement such that lesions which enhance on at least 2 consecutive MRI scans have lower MTR values than lesions which only enhanced on the first scan (117). If loss of myelin decreased MTR values, then remyelination may restore normal MTR values (115). Furthermore, newly gad-enhancing active white matter lesions have significantly greater MTR values than ring-enhancing lesions which are typically older and may be a reactivated lesion. The central portion of the ring-enhancing lesions have lower MTR than the enhancing portion, probably due to the chronicity of the central portion and therefore a greater amount of damage in that region as compared to the gad-enhancing area (118). The average MTR of a white matter lesion will drop as a lesion begins to enhance but can partially or completely recover in the subsequent 1-6 months (117), which is likely a result of edema and its resolution after repair of the blood-brain barrier in combination with the formation of a glial scar, and demyelination and remyelination (119). Some of the low MTR regions may not have demyelinated, in which case the resolution of the edema, microglial activation, and potentially axonal swelling would also result in a fluctuation in MTR values. It was reported that 38-44% of
T2-positive lesions had an increase in MTR at a 6 month follow-up, 5-12% had decreased average MTR values and about 50% remained the same (109, 120). This evidence suggests that the longer a white matter lesion enhances, the more severe the tissue damage will be, likely resulting in a T1 black hole and reduced MTR values. However, only a small percentage of white matter lesions will have rapid progressive structural damage shortly after formation since only 5-12% had decreased MTR values 6 months later. For the majority of lesions, it is likely that they stabilize for a period of time and slowly accumulate axonal loss as the chronic demyelinated axons are exposed to inflammatory factors and potentially harmful molecules such as reactive oxygen species. MTR changes are also observed in normal appearing white matter of MS patients (121, 122). This reduction of the MTR values in normal appearing white matter is likely a result of the abnormally thin myelin observed in biopsies from MS normal appearing white matter (123) or results from the axonal loss that also occurs due to degenerative mechanisms (124, 125). Axonal changes such as axonal loss and swelling in T2T1MTR regions were the major pathological feature in the absence of demyelination (47), which is an important observation in that demyelination is not the only pathology to effect MTR. This evidence is of particular interest with respect to the myelocortical MS patients, where white matter remains unaffected by demyelination. It is possible that the axonal changes described above may have also occurred in the white matter of the myelocortical MS patients without demyelination. Disease duration correlates with decreases in MTR values in normal appearing white matter and in white matter lesions (126). This would suggest that pathological changes accompanying loss of myelin and axons may be independent of the presence of peripheral immune infiltration. This supports the possibility that a neurodegenerative process decreases MTR values of normal appearing white matter. Despite the extensive changes and damage occurring in the cerebral
white matter, disability correlates weakly at best with these measurements of damage, providing further support for demyelination and damage resulting elsewhere in the central nervous system which also significantly contributing to the neurological dysfunction.

As previously mentioned, MS is not strictly a white matter disease as it was once believed to be, the gray matter can as be affected, but gray matter lesions are not visible macroscopically or detected by the conventional MRI techniques discussed previously. This is in part due to the lower levels of myelin in the gray matter and in the case of the cortex, the close association with the cerebrospinal fluid (resulting in partial voluming effects), as well as the lack of infiltrating peripheral immune cells, or disruptions to the blood-brain barrier. Therefore, other MRI modalities have been utilized to increase the detection of the gray matter lesions, such as double-inversion recovery (DIR) and magnetization transfer ratio (MTR) or by the use of a higher magnetic field (7T).

Double-inversion recovery has been shown to detect leukocortical lesions and intracortical lesions better than subpial cortical lesions (127). Geurts and colleagues showed that even with 3D FLAIR MRI (a conventional MRI technique), only 5% of cortical and 38% of deep gray matter lesions were visible compared to the histological examination of postmortem tissue (52). However, with the 3D DIR sequence, roughly 18% of cortical lesions were detected, which is 1.6-fold higher detection rate than 3D FLAIR (127). Detection of cortical lesions by non-conventional MRI techniques is currently not included in diagnostic criteria of MS or in any outcome measures for MS drug trials. As the detection rate improves cortical and deep gray matter lesions will likely be added as evidence for MS diagnosis and as outcomes in testing drug efficacy. The current detection limits of only a small number of cortical lesions is still important, particularly to be able to perform the same MRI-histopathological studies that have been
executed on white matter lesions. When cortical lesions are detected by MRI, cortical lesion volume has been shown to be increased in cognitively impaired MS patients and correlates better than white matter damage with cognitive deficits in RRMS (128). Cognitive and physical disability correlate with cortical lesion volume measured by 7T MRI better than white matter lesion volume; however, type III subpial lesions had the strongest relationship to disability compared to the other lesion subtypes (129). Recent studies have shown that, in some MS patients, cortical lesions may actually precede white matter demyelination (130-132) and can be an early event (7), even though many studies have described them as occurring more in the progressive stages of the disease (133). Furthermore, the magnitude of cortical demyelination does not correlate with the extent of focal white matter demyelination (134) nor does the amount of cerebral gray matter lesions correlate with white matter lesions. This could possibly be due the small sample sizes in the studies (n=6) or it may be related to the stage or subtype of MS patients in the study.

Multiple sclerosis patients show reduced MTR values in the gray matter compared to healthy controls, suggesting that demyelination occurred in the gray matter (135, 136), which inversely correlates with the clinical disability severity (r= -0.65, (137). Interestingly, there is a correlation between gray matter MTR abnormalities and T2-lesion volumes (136-138) which leads to the possible conclusion that a portion of gray matter damage may be a secondary event to the retrograde degeneration of fibers in the white matter lesions (21). When the mean MTR values are compared across MS subtypes, there is a pronounced increase in gray matter abnormalities from RRMS to SPMS and PPMS (139, 140). These changes in gray matter MTR can be observed even in the CIS patients in regions such as the thalamus, lenticular nucleus, head of the caudate and insular cortex (141), demonstrating that early pathology develops in the gray
matter alongside the more readily detectable white matter lesions. MTR measures of the cortex are significantly lower in regions with intermediate amounts of demyelination and complete cortical demyelination compared to myelinated cortex, suggesting that MTR is sensitive to the demyelination within the cerebral cortex (142).

MRI changes in the cerebral cortex have been shown to be associated with the progression of disability in MS patients, such that patients with stable cortical pathology progressed very little and those with continuous accumulation of cortical pathology progressed the most (143). Cortical lesion loads not only correlate with physical disability but also with level of cognitive impairment, which developed in MS patients with higher cortical lesion loads compared to MS patients that were cognitively normal (144). Cortical reorganization following a lesion is thought to be important for two reasons; the first is that the plasticity of the cortex may in part be responsible for the discordance between disability measurements and pathological quantifications, and the second reason is that this reorganization may be a limitation of functional repair (52). It has been suggested that the cortical lesions, most likely subpial lesions, in concert with cortical atrophy, might play a role in the pathogenesis of cognitive impairments, and some of the other neuropsychiatric symptoms that commonly occur in MS patients including depression, dementia, seizures, and fatigue in MS patients (51, 52, 145). Cognitive impairments such as memory loss or slower processing of information are seen in 40-60% of MS patients, which can greatly affect these individuals’ quality of life. In a longitudinal study over a 3-year period there was a significant relationship between the number of cortical lesions by DIR MRI at the time of follow up and measures of visuospatial memory and processing speed (146).

Deep gray matter demyelination is also difficult to detect due to the lower levels of myelin and for some regions such as the thalamus and hippocampus due to their proximity to the
ventricles creating partial voluming affects with the CSF. There are limited MRI studies that focus on deep gray matter demyelination, but this demyelination has been demonstrated histologically in the cerebellum (81), thalamus (81, 82, 147), hippocampus (56, 79, 147, 148), caudate (82), putamen (82), pallidum (147), claustrum, hypothalamus (82, 149), amygdala and substantia nigra (45). Most of the MRI studies of deep gray matter regions performed analysis of the atrophy occurring in these regions, which is discussed later in this chapter but little is reported on MRI lesions in these regions. However, one group of MS researchers, has shown that the average diffusivity measurements are decreased in MS patients compared to normal controls, but also that the decrease is greater in SPMS patients compared to RRMS and PPMS patients, suggesting that this destruction of the gray matter accumulates over the course of the disease (150).

In addition to cerebral gray matter demyelination, spinal cord gray matter is also frequently affected, but is difficult to distinguish from spinal cord white matter by MRI. Common sensory symptoms in MS patients such as pain, headache, fatigue, vertigo, transient neurological events, and Lhermitte’s phenomenon may develop from gray matter spinal cord lesions or cortical lesions in associated areas (145). The gray matter seems to play a significant role in the progression of the disease and the accumulation of disability. How much spinal cord gray matter pathology contributes to disease progression and disability has yet to be determined.

Spinal cord lesions can also be observed on conventional MRI scans, but unlike brain white matter lesions, spinal cord lesions generally do not gad-enhance (151). Imaging of the spinal cord is rarely performed due to the difficulties with artifacts from movement of the lungs, the heart beating, large vessels and truncation artifacts; these ultimately result in suboptimal resolution and failure to detect small lesions. However, spinal cord imaging can aid in the
diagnosis of MS by demonstration of lesions that are disseminating in time and/or space since brain and spinal cord lesions do not have to occur simultaneously and approximately 5% of clinically definite MS patients have normal brain MRI scans but show spinal cord abnormalities by MRI (151). On average patients with brain MRI scans negative for lesions have at least 2 spinal cord lesions, as demonstrated in the 20 MS patients from a study by Thorpe and colleagues (152). Spinal cord lesion volumes do not correlate with brain lesion volumes, which suggests that these two central nervous system regions develop lesions independently from one another (153). Not only lesions are detectable in spinal cord imaging, similar to the brain, diffuse abnormalities are also detected, but lesions or diffuse abnormalities in the brain by MRI do not correlate to the same pathology in the spinal cord and vice versa (154). It is estimated from MRI studies that 74-85% of MS patients develop focal spinal cord changes; however, when focal and diffuse cord abnormalities are accounted for, 90% of MS patients have some type of cord abnormality (as reviewed by (151)). Further heterogeneity is demonstrated by the observation that spinal cord pathology type varies greatly between the types of MS with respect to lesion load, atrophy and normal-appearing cord damage. Atrophy is not as prominent and diffuse signals are usually absent in RRMS spinal cord (154), but rather they tend to exhibit with more focal lesions. Spinal cord lesions are most frequent in the cervical cord and when these lesions were examined in RRMS patients, no atrophy was present in the gray matter portion of the cord. Interestingly, the cervical gray matter had a lower magnetization transfer ratio (MTR) compared to controls which, correlated with the degree of disability in the patients (155), suggesting demyelination within this region. In SPMS spinal cords, the lesions observed are more extensive and confluent, along with an increase in atrophy accumulating at this stage (154, 156). MRI scans of SPMS patients also show normal-appearing cord damage, which likely adds
to the disability of a patient, but is difficult to really quantify with the lesion pathology (156).
Furthermore, examination of spinal cord lesion load and functional disability by functional MRI (fMRI) revealed that the severely disabled MS patients demonstrated an enhancement of cord activity compared to the controls and less disabled MS patients (156). The fMRI changes did not correlate with the T2-visible cord lesions, which may suggest that these changes are in fact due to the gray matter injury not visible by MRI (156). SPMS and PPMS patients are more likely to develop a diffuse abnormal signal in the spinal cord. The MS patients with this diffuse signal displayed a significantly smaller mean spinal cord cross-sectional area compared to the patients without the diffuse abnormal signal (151). This observation would suggest that the normal-appearing spinal cord damage occurs in both progressive forms of the disease and likely contributes to spinal cord atrophy (156, 157). Interestingly, PPMS patients have been shown to be more likely to develop the diffuse cord abnormalities with or without the presence of focal lesions, possibly suggesting a more neurodegenerative process with little inflammatory involvement (157). PPMS patients are also more likely to have completely normal brain MRIs (152) or a reduced brain lesion load compared to SPMS (158), meanwhile having a greater percentage of their total lesion load in the spinal cord (159). Although spinal cord damage correlates more strongly with disability than brain lesion load, it still does not account for all of the accumulated disability, especially the cognitive impairments.

Brain atrophy is prominent in the later stages of MS, but is also present in RRMS patients with short disease duration and minimal brain lesion volume, for both gray and white matter (160, 161). Whole brain atrophy is another MRI measurement frequently utilized in clinical trials for an outcome measurement of drug efficacy. This type of measurement can be performed with different methods which range from those performed manually to semi-
automated to completely automated techniques, as well as cross-sectional studies making comparisons between MS patients and healthy controls or longitudinal studies within MS patients over a fixed period of time, which makes comparing different trial data difficult if differing methods were implemented. Atrophy measurements are complicated due to the requirement of 3D coregistration of MRI scans, which basically aligns the scans so that identical brain regions from different scans line up together in order to identify any changes occurring both regionally and globally. This coregistration can be confounded by differences in the slice position of the scan between patients, or head orientation at the time of the scans over different time points, partial volume effects, motion effects or upgrades of the scanner, ultimately making for a substantial amount of post-processing of the images for the measurement of change in whole brain volume (21). Another confounding factor is the pseudo-atrophy that results from the infiltration of the immune cells and edema which will initially increase the brain and/or lesion volume and with the resolution of these pathologies there will appear to be an overall decrease much larger than the actual brain tissue volume decrease (162). However, it is clear that neuronal and axonal damage is occurring within lesions and normal appearing tissue and that this damage is the pathological substrate of the progressive, irreversible disability, which makes it an objective measure of the global disease burden of the central nervous system and possibly an indirect measure of the overall disease severity as well. Therefore measuring the loss of these structures is becoming a focus of MS research and drug trials (21). The loss of the axons, neurons, and oligodendrocytes in addition to the chronic demyelination and gliosis contribute to the volume reduction of the parenchymal tissue and expansion of the ventricles and space within the sulci. One commonly used technique for measuring whole brain atrophy is brain parenchymal fraction (BPF) which is calculated by segmentation of the brain parenchymal
volume from the CSF and other background from the cranial structures. One of the major advantages to BPF is that this measurement is normalized to skull size of the patient and not normalized to an average brain from controls. This brain parenchymal volume is then divided by the brain parenchymal volume plus the volume of the CSF as show below (104).

\[
\text{Brain Parenchymal Fraction} = \frac{\text{Brain Parenchymal Volume}}{\text{Brain Parenchymal Volume} + \text{Cerebrospinal Fluid Volume}}
\]

The brain parenchymal fraction can be compared across patients and controls or within patients across the disease duration to understand how it is changing due to the disease or to the administration of MS therapies. Whole brain atrophy is detectable in CIS patients, ventricular enlargement, which was significantly greater in MS patients that progressed to clinically definite MS within a year compared to those that did not develop MS (163). In RRMS patients, a loss ranging from 0.5-1.0% of brain tissue volume per year compared to healthy controls was observed (104, 137, 164-166), these changes have also been detected by central slab volume (167), ventricular volume (164), and for specific regions such as corpus callosum (167), brainstem (168, 169), and cerebellum (168) in cross-sectionals studies. In a clinical trial for glatiramer acetate (an approved MS drug), the change in brain volume over a 9-month period within patients correlated with the number of gad-enhancing lesions at baseline specifically for the placebo group (170). This correlation is intriguing because it provides evidence for a connection between the early infiltrating inflammation and the eventual brain volume loss. Furthermore, RRMS patients followed for 3 years with monthly MRI scans demonstrated a correlation between changes in brain volume that were preceded by changes in gad-enhancing lesions by 3-6 months, suggesting the atrophy lags behind the brain inflammation (171). In
addition, the number of gad-enhancing lesions, at year 2 of an 8 year follow up of 106 RRMS patients, was a significant predictor of the change in brain parenchymal fraction from year 2 to 8 (105), as have the presence, and volume of gad-enhancing lesions at baseline (100, 162, 172, 173). These studies suggest that the more active inflammatory processes result in greater brain volume loss, but that inflammatory lesions do not account for all atrophy that accumulates. Enhancing lesions are not the only lesions which correlate with atrophy, T2 weighted lesions and the changes in these lesions over time do as well, suggesting that the focal changes progress into atrophy (100, 174, 175). Interestingly, the T2 lesion volumes only attributed to 27% of the variance in the subsequent brain atrophy (105), meaning that although lesions contribute to the brain volume loss, other pathological mechanisms are also resulting in the atrophy measured by MRI. Furthermore, whole brain MTR correlates with brain atrophy (r= 0.6-0.7) (176) better than lesions, which also gives evidence for tissue damage playing a significant role in atrophy accumulation and that both atrophy and MTR measurements may be sensitive to the same pathological substrates of diffuse damage in the normal appearing tissue (177).

Brain atrophy is also prominent in the later stages of the disease, with similar atrophy rates occurring in the SPMS patients as was observed in the RRMS (137, 177, 178). However, in SPMS patients with a more rapidly progressing disease course, brain atrophy rates were increase to 1.9% loss of brain volume per year despite suppression of the inflammation with transplantation of autologous hematopoietic stem cells (179). This was also demonstrated in longitudinal studies that these patients with increase atrophy rates have a higher probability of worsening clinically (162, 180). In fact, after an 8 year follow up, 56% of the MS patients with significantly higher atrophy rates in the first 2 years of the study reached an EDSS score of 6, whereas only 24% of the patients with lower atrophy rates reached the same level of disability.
In addition, brain atrophy levels have been shown to be identical between SPMS and PPMS patients with the same disease duration (177), or similar disability levels, even despite significantly lower lesion loads in the PPMS patients (170), which suggests that regardless of relapses, white matter lesions or disability, patients develop roughly the same amount of brain volume loss. This is further demonstrated by the results of a longitudinal study in which reductions in brain parenchymal fraction, as well as an increase in ventricular fractions were no different between the RRMS, SPMS and PPMS patients (164, 177), despite the known higher levels of inflammation in RRMS patients over the progressive forms of the disease.

As mentioned earlier, another possibility is that brain atrophy is not a direct result of the infiltrating peripheral immune cells but rather is a process primarily of diffuse axonal damage (181), or that atrophy is initially a large component of inflammation, but that this relationship changes over the course of the disease with inflammation having a smaller effect on the overall atrophy occurring (21). If the axonal damage is to be attributed to the other 73% of brain atrophy variance, then there should be a strong link between disability and atrophy. Atrophy is found to correlate better with disability than the weak correlation with white matter lesion load, possibly due to the lesion load by MRI excluding the gray matter lesions while total brain atrophy does not. In several different cross-sectional studies of MS patients, EDSS moderately correlated with brain atrophy (r = 0.2-0.5) (104, 164, 165, 168), the correlation may have been stronger if spinal cord atrophy was included since in the higher range of the EDSS scores it is highly weighted on ambulation, but does not account for the cognitive changes which would be affected by brain volume loss. The ability of the brain to compensate for the damage to neurons and their axons early in the disease, due to the functional reserve and remaining capacity for tissue repair may also create a dissociation between the atrophy and disability accumulated (21).
Gray matter atrophy has been shown to commence early in the disease course (182), and contribute equally to whole brain atrophy as white matter atrophy (160). Gray matter atrophy, particularly in the precentral gyrus, superior frontal gyrus, thalamus and putamen, was predictive of disease progression from clinical isolated syndrome to clinical definite multiple sclerosis (183). Gray matter loss was significantly greater in the pre- and post-central gyrus in RRMS patients compared to clinical isolated syndrome patients and greater still in cortical regions of frontal, parietal, temporal and occipital lobes, cerebellum, the superior and inferior colliculus and deep gray matter structures in SPMS compared to RRMS patients (184). Gray matter fraction is significantly decreased compared to controls in clinically isolated syndrome patients (185) and RRMS patients (186), but white matter fractions are only decreased in a subset of MS patients, which suggests that the gray matter damage begins early in the disease and likely exceeds that of the white matter damage (187-189) similar to the demyelination. Fisher and colleagues (160) demonstrated that “an increasing contribution of gray matter tissue loss to whole-brain atrophy as MS advances,” after an 8-year follow-up. However, this suggests that at the beginning stages, gray matter atrophy may be secondary to the white matter tissue damage, yet as the disease progresses the gray matter tissue loss becomes independent of the white matter tissue damage. This is further demonstrated by the stronger correlation between gray matter atrophy and T2 lesion volumes ($r= -0.43$ to $-0.73$) than with white matter atrophy and the T2 lesion volumes (137, 188, 190, 191). Whereas, measurements of whole brain atrophy correlates strongly with disability, imaging of gray matter atrophy does as well, indicating that gray matter tissue damage may be a dominate pathology that cultivates into neurological disability (160, 187). Gray matter atrophy not only correlates with physical disability (192), but has been shown to be significantly
greater in individuals with cognitive impairment, particularly deficits in verbal memory, verbal fluency and attention/concentration, compared to non-cognitively impaired MS patients (193).

Cortical atrophy is defined as a loss of gray matter that results in a thinning of the cortical ribbon by MRI measures. Similar to gray matter atrophy, cortical atrophy has been correlated to many MS-related factors such as physical and cognitive disability and disease progression (194-198). Several researchers have demonstrated that MS patients with progressive disability have greater cortical atrophy than MS patients who were clinically stable (143, 144). In addition, the mean cortical thickness was shown to inversely correlate with the total white matter lesion load, EDSS, and age, but with regional variations. However, atrophy does not affect the cortex in a linear fashion but rather accumulates in a region specific manner. The anterior portion of the cingulate gyrus being the area most affected followed by the posterior insular cortex, the prefrontal, temporal and parietal association cortical areas, pre- and post-central gyrus, but not in the visual cortical regions (161, 194). There are a few possibilities to explain the region specific accumulation of cortical atrophy, the first being a result of axonal transection in white matter lesions that project to or originate from cortical areas with demyelination, resulting in Wallerian degeneration of the cortical axons. This would suggest that the location of white matter lesions would leave specific cortical areas more vulnerable to atrophy. For instance periventricular white matter contains tracts to and from the prefrontal, cingular, and other association cortical areas, when a lesion forms in this white matter region, it would leave those cortical areas more vulnerable to Wallerian degeneration and thus atrophy (44). Another explanation could be that the gray matter damage and cortical atrophy develops completely independent of the white matter damage, however this would be difficult to demonstrate due to the early and continued presence of white matter lesions in MS patients. Although it has been shown that the best
predictors of gray matter volume were measurements of neuronal density, neuronal size and axonal density, however no correlation was observed between cortical volume and myelin density or white matter lesion load (199).

Spinal cord atrophy is fraught with the same difficulties as spinal cord lesion imaging, movement artifacts due to breathing and the heart beating, and a small imaging target leads to this region rarely being imaged in MS patients. The cervical spinal cord is most often the region of the cord imaged because of its wide expansion and it does not rest directly under the heart and lungs. The atrophy measured by a reduction in the cross-sectional area that occurs in the spinal cord is also been shown to be greatest in cervical cord lesions compared to lumbar cord lesions (200), making the cervical cord the best region of spinal cord imaging. Spinal cord atrophy can be detected early and may be present prior to the onset of clinical symptoms (201), which is made evident by the data from a study of clinically isolated syndrome patients with abnormal brain MRIs, 74% had significantly decreased cervical cord areas. In RRMS patients a 40.8% mean volume reduction was found in the upper cervical cord (168), however other studies in RRMS have found no differences between healthy controls and RRMS cervical cord areas (202-204). The differences in the results observed in these studies could have been due to the differences in the sensitivity of the methods or in the patient population chosen for analysis, as some patients progress faster than others. During the early stages of MS, the atrophy measurements may represent the underlying tissue damage occurring between each relapse, which currently cannot be measured by clinical examination due to the plasticity capacity of the central nervous system. Several studies have agreed that there is significantly more spinal cord atrophy in the SPMS patients than in the earlier stages of MS (168, 202, 203, 307). Interestingly, PPMS patients show similar amounts of spinal cord atrophy as RRMS with similar disease
durations (202), suggesting that atrophy accumulates at a similar rate for the different MS types. In the same study, it was shown across all the MS patients (RRMS, SPMS, and PPMS) that the mean annual cord atrophy rate was -3.7%, which likely contributes greatly to the EDSS scores, probably due to the fact that EDSS scores are strongly weighted on ambulation. MS patients that had developed spinal cord atrophy were more disabled than patients that did not have spinal cord atrophy (205), which demonstrates the contribution of spinal cord pathology to overall disability. The correlations between EDSS and spinal cord atrophy have not only been shown to be more consistent across studies, but are also stronger ($r = 0.5 - 0.7$), than correlations with lesions or brain atrophy (154, 203, 206). Atrophy in the spinal cord is mostly a global loss of axons due to degeneration and not a loss of tissue within the focal cord lesions (207), with an average of 60% decrease in axonal density and 68% loss of axons throughout the spinal cord lesions compared with controls (54). The reduction in cross-sectional area affects the white matter and gray matter relatively equally; hence the white to gray matter ratio remained similar to controls (54), however the tissue loss in the early stages of the disease is primarily within the white matter of the cord and the gray matter loss occurs later (208). The precise pathological mechanism of atrophy is not known and may not be specific, but different combinations of pathological events can result in the tissue loss. The exact pathological cause of atrophy does likely represent an irreversible tissue loss due to these pathological mechanisms. In the later stages of the disease new lesions are less frequent, therefore it has been suggested that lesions likely don’t contribute to the accumulation of tissue loss to a significant degree, but rather tissue destruction and degeneration independent of the infiltrating inflammation (21). More likely it is a complex relationship between inflammatory events, tissue damage and the resulting atrophy.
5.1.7 Disease-Modifying Therapies for MS

There are currently 14 approved drugs on the market for MS treatment. Here we discuss a select few with regards to their efficacy, which phase of MS they are prescribed for, whether they are used as a first line of defense or are more aggressive therapies, and the negative side effects of each one.

The first of a long list of disease modifying therapies for MS was the drug Interferon beta Ia (Avonex and Rebif) or Ib (Betaseron and Extavia), which are cytokines that are normally secreted by immune cells to prevent the replication of viruses. These cytokines also have an anti-inflammatory response that appears to be effective for dampening the aberrant immune response in MS patients (209). Unfortunately, the exact mechanism of action for these beneficial effects is unknown as to whether it is strictly the anti-inflammatory mechanism that results in the therapeutic response or if the anti-viral effects also play a role. The Interferons have been shown to reduce the number of relapses for MS patients and can reduce the number of lesions by MRI between 50 and 80% (86, 210). Despite the clear beneficial effects on the relapses in MS patients the disease still progresses onto the SPMS stage, suggesting that strictly regulating the immune system may not be the only disease mechanism at play. Interferon beta Ia and Ib are used as a first line of defense drug.

Glatiramer Acetate (Copaxone), another first line of defense therapy approved for the RRMS phase of the disease, is a mixture of L-glutamic acid, L-lysine, L-alanine and L-tyrosine which form a copolymer polypeptide that resembles myelin basic protein (MBP), a structural protein in the myelin sheath (209). Again the exact mechanism of action is unknown for this
drug, but is believed that this drug inhibits suppressor T cells that are activated in the periphery and travel to the central nervous system and cause damage including demyelination. Although glatiramer acetate showed a decrease in the number of relapses and slowed decline into the progressive phase of the disease (as reviewed by Freedman (211, 212)), many patients cannot tolerate this treatment and its aversive side effects, so therefore they are forced to try a different medication.

Suppression of the aberrant immune response seemed to the pharmaceutical companies to be the ultimate strategy for treating MS, and thus Mitoxantrone, a treatment originally produced to treat specific forms of cancer was quickly approved for MS. This drug dampens the activity of lymphocytes and macrophages which are likely disrupting and destroying the myelin sheaths. It accomplishes this action by interfering with antigen presentation and secretion of pro-inflammatory molecules like INFγ, TNFα, and IL-2 (209). The effects on the immune cells are most likely mediated through its ability to insert itself into the DNA of a cell and inhibit topoisomerase II from repairing the DNA damage (213). This drug was shown to reduce the relapse rates in RRMS patients and to slow progression to SPMS, but this treatment can only be used for 2-3 years, due to the severe aversive side effects that develop with chronic use. Therefore, it is used as a more aggressive approach and is not as a first line of defense drug.

Natalizumab (Tysabri) is another immunomodulatory drug approved for the treatment of MS patients, but again is used to treat MS patients that do not respond to the first line of defense drug therapies. This recombinant humanized immunoglobulin monoclonal antibody binds to the alpha 4 subunit of the very late activating antigen 4 integrin, which is expressed on the surface of leukocytes to inhibit their adhesion to endothelial cells through the interaction with VCAM-I receptors expressed on their surface (209). Prior to use of this drug the patient must be tested for
the John Cunningham virus (JCV) by analysis of the cerebrospinal fluid, which increases the patient’s risk of the brain infection progressive multifocal leukoencephalopathy (PML) that often leads to death or severe neurological disability. Long-term use of Natalizumab can also increase an individual’s risk of developing PML; therefore, the patient must be monitored closely during its administration. Reductions in relapse rates by 54-67% were observed in patients on Natalizumab compared to a placebo control group. Although this percentage may seem low, this includes the MS patients that failed all other MS therapies and therefore any efficacy for these patients is substantial. When combined with interferon beta-1a, the annualized relapse rate is lower than that of interferon beta-1a alone (214). As with the other approved MS drugs, Natalizumab does not appear to have any effects on EDSS scores in SPMS patients, and therefore it is only used during the RRMS stage of MS (215).

Fingolimod is an orally administered drug which dampens the immune system by inhibiting migration of lymphocytes from the lymph nodes, therefore it is hypothesized that the mechanism of action is in reducing the number of lymphocytes gaining access into the central nervous system (209). Multiple studies have shown this drugs ability to reduce the number of relapses as compared to both placebo and interferon beta-1a (Gilenya 2011; (216-218). Similar to Natalizumab, Fingolimod increases a patient’s risk of infections since the lymphocytes cannot exit the lymph nodes to sites of infection (209). Furthermore, patients must be observed for 6 hours following their first dose for bradycardia which is a common side effect.

None of the above mentioned drugs is curative for MS, but merely shortening the duration of relapses, decreasing the number of relapses and lengthening the time between relapses. All of these medications have adverse side effects including flu like symptoms, pain and redness at injection sites, liver dysfunction, depression, headache, etc. So the medications
may treat aspects of this disease, but it is not a cure and the side effects are not something that can easily be ignored. Many clinical trials have emphasized the importance of early treatment of MS patients in the early inflammatory-mediated relapsing stage of the disease in order to delay the onset of progressive MS. Of the MS patients in the placebo arm of these trials that remain untreated, approximately 85% of them will reach the McDonald criteria of MS within 2 years of their initiating event (219). Neurological symptoms occurring during acute relapses are often initially managed by the use of corticosteroids to maintain function and improve the patient’s quality of life. However, the corticosteroids are only a brief fix, and eventually another attack will occur or the patient will progress into the next stage of the disease where there is currently no FDA approved drug specific for the progressive stages of the disease. Although there have been several drug trials focusing on Primary and Secondary Progressive MS, none have shown true efficacy of delaying time to confirmed disability progression or evolution of brain atrophy, suggesting that a neuroprotective agent may be more beneficial at this stage or even prior to this stage, since there is “a dissociated effect of anti-inflammatory treatments on disease activity and disease progression,” for the progressive MS patients (213). A need to understand the underlying mechanisms at this stage of the disease is necessary for targeting therapeutics to this stage of the disease. Through the examination of postmortem MS tissue, insights into disease pathogenesis may lead to an understanding of what types of therapeutics would be most beneficial for the progressive stages and maybe even the earlier stages of MS to slow or completely stop the progression of this devastating disease.

Research continues to identify other possible therapies for both the relapse-remitting phase of the disease but also for the progressive stages as well. In addition to the above mentioned drugs, other potential therapies for MS patients that are gaining in popularity are
related to increasing remyelination mechanisms, and neuroprotection. One such therapy is the monoclonal Lingo-1 antibody that Biogen pharmaceuticals currently has in a phase II trial as a potential treatment to induce remyelination by blocking the inhibition of Lingo-1 protein on oligodendrocytes. This therapy is discussed in further detail in the final chapter.

Neuroprotective drug therapies seem to be the most attractive drug target for progressive MS patients; however, one of the reasons this type of treatment is harder to get approval for is because of the outcome measures, such as brain atrophy or conversion rate of new lesions to T1 black holes, have to be followed for extended periods of time (about 3-5 years) to prove efficacy (21). A few candidate drugs for this category would be recombinant human erythropoietin (EPO), sodium channel blockers, neuroimmunophilin ligands, and glutamate antagonists.

Erythropoietin (EPO) may seem like a strange choice for neuroprotection, since it is an endogenously made molecule by the kidneys in times of low blood oxygen levels to activate red bone marrow to produce more red blood cells. However, receptors for this molecule are also found in the central nervous system and has already been shown to be neuroprotective in animal studies for hypoxia, hypoglycemia and growth factor deprivation, spinal cord injury, and free radical injury (285-289). It is thought that EPO has anti-apoptotic action that prevents axonal degeneration (290) by activating Janus kinase 2 (JAK2) leading to a cascade resulting in the transcription and translation of pro-survival molecules (287). Erythropoietin has also be administered to mice with experimental autoimmune encephalomyelitis (EAE), which reduced the overall severity of the impairment, decreased inflammation infiltration, and reduced demyelination and axonal damage (291). All this evidence suggests that EPO may be a promising potential therapy for MS patients.
Sodium channel blockers are thought to be a potential therapy because they would prevent the accumulation of sodium within the axon which causes a reversal of the sodium/calcium exchanger, leading to increased levels of intracellular calcium and ultimately devastating events inducing axonal degeneration. Two such channel blockers phenytoin and flecainide have already been shown to be protective against axonal damage and have improve clinical outcomes in the EAE mouse model of MS (292). One potential side effect of these types of therapies is that it would likely delay the recovery of relapses, since conduction would be blocked in axons that had a redistribution of the sodium channels along the demyelinated axon (21).

Chaperone proteins in neurons called neuroimmunophilin ligands have been tested in mouse models of both stroke and excitotoxic neuronal death and have shown promising results (293, 295). The neurommunophilin ligands are thought to be protective of neurons by either upregulating the antioxidant glutathione or through regulation of steroid hormone receptors on central nervous system cells (294). In EAE, the neuroimmunophilin ligand FK506 was shown to reduce axonal injury when administered at the onset of paralysis (296).

Glutamate antagonists may be beneficial both to axons as well as oligodendrocytes, since excessive amounts of glutamate, from neurotransmission and release from microglia (303) in the synaptic cleft and extracellular space could be over-activating the AMPA receptors on axons and oligodendrocytes. These glutamate antagonists would block both AMPA and kainite receptors which has been shown to decrease axonal injury (305). One trial of riluzole in primary progressive MS patients demonstrated a slowing of cervical cord atrophy in the patients on this glutamate antagonist compared to the placebo group (304).
It is likely that a combination of the anti-inflammatory drug therapies with neuroprotective and/or remyelination capabilities will become the standard care for MS patients. More research is necessary to understand the mechanisms of action for these potential drugs, as well as research to elucidate disease pathogenesis.

5.2 Conclusion of Review

Multiple sclerosis is a devastating disease of the human central nervous system which arises due to a combination of genetic and environmental factors resulting in demyelination from an aberrant immune response and ultimately neurodegeneration. Millions of individuals worldwide are affected by this cureless disease, making the research for a curative or preventive therapy so necessary. Current therapies target the abnormal immune response in these patients; however, this type of therapy is only effective in those patients with the relapsing-remitting stage of the disease. These disease modifying therapies are partially effective at reducing the number of relapses in patients and at preventing newly forming demyelinating white matter lesions, which are the hallmark of MS, however little is known about their effect on spinal cord, cortical and deep gray matter demyelination.

MRI is an integral part of our evaluation of disease progression particularly with respect to clinical trials as well as in the diagnosis for this disease and clinical course for the various MS subtypes. The course and the pathology of MS are quite variable, which demonstrates the complexity of this disease. The white matter lesions result from infiltration of peripheral immune cells that attack myelin and oligodendrocytes leading to demyelination. The
demyelinated axon is left vulnerable to the surrounding active inflammatory microenvironment in chronic active lesions and long term microglial activation. Gray matter demyelination develops with far fewer peripheral immune cells present, suggesting the possibility of a different mechanism causing the pathology within these regions. Through the examination of the white matter and gray matter pathology from postmortem MS specimens we can gain a better understanding of how this disease develops and therefore develop more effective therapeutic targets to slow or prevent the progression of MS.

5.3 Research Questions and Hypotheses

There are still many unanswered questions related to the exact mechanisms of the pathogenesis of MS and therefore what the best course of action to take in developing a therapeutic which will slow the progression of this disease. As described earlier, there are several different variants of MS; how each of these variants arises is unknown. One possibility is differences due to genetics or environmental factors playing a role in the type of disease course. In addition, what leads to the differences in demyelination and remyelination of the gray and white matter of the central nervous system, as discussed earlier in the section on gray matter pathology. Are these mechanisms of demyelination and neuronal/axonal loss the same or is there a different mechanism underlying the different types of pathology and is this location-specific? The underlying mechanisms are of the utmost importance, because they will determine the best therapeutic pathway which will be effective not only in the early inflammatory phase of the disease, but also in the later progressive stage.
As discussed previously, the mechanisms of white matter demyelination have been studied extensively due to their identification both by MRI and macroscopically on postmortem tissue, as well as modeled in mice; however, the mechanisms of cortical demyelination, particularly in subpial lesions, are unknown. The mechanisms involved in the demyelination of the cortex may be a result of primary demyelination by an as-yet unidentified mechanism. Another possible mechanism is the peripheral inflammation traffics to the leptomeninges, forming ectopic follicles which could contribute to demyelination the cerebral cortex. Cortical demyelination could be produced secondary to demyelination and injury occurring in the cerebral white matter. Examination of cortical demyelination without the presence of cerebral white matter lesions would determine the probability of cortical demyelination being a secondary event to the damage of the white matter lesions. In our rapid autopsy tissue collection, 12 of the 97 MS patients were devoid of the hallmark cerebral white matter lesions upon gross inspection of 1cm thick coronal brain slices. These patients showed MRI abnormalities indicative of MS pathology, typical MS patient clinical histories, and upon gross inspection of the spinal cord typical discolorations consistent with demyelination. Through immunohistochemical analysis of the cerebral cortex, revealed cortical demyelination as another substantial pathology in addition to the spinal cord lesions. These patients will be referred to as myelocortical MS and were investigated to understand the presence of cortical and spinal cord demyelination in the absence of cerebral white matter lesions.

The major questions that were examined in this thesis work are the following:

Can cortical, spinal cord and deep cerebral gray matter demyelination occur in the absence of cerebral white matter demyelination in MS patients? Is the mechanism of cortical, spinal cord and deep cerebral gray matter demyelination similar as those in MS cases where
lesions did occur in the cerebral white matter? Based on the previous work discussed in this chapter, it was hypothesized that cortical, spinal cord and deep gray matter lesions would be present in MS cases that were lacking cerebral white matter lesions. This hypothesis will be tested by quantifying demyelination in these commonly affected regions of the central nervous system in the unique cohort of myelocortical MS patients essentially devoid of cerebral white matter lesions and comparing them to classical MS brains containing abundant macroscopic cerebral white matter lesions.

Another intriguing question is what specific pathological alterations, in the absence of demyelination, are resulting in MRI white matter abnormalities in myelocortical MS patients? Are the pathologies different with different MRI categories of lesions? From the literature, it is known that axonal swelling, demyelination, gliosis and disruptions in the blood-brain barrier can all contribute to abnormal MRI signals within the white matter; however, as Fisher and colleagues have shown demyelination is not necessary for the presence of a MRI white matter lesion (47). Therefore, we hypothesized that the MRI white matter abnormalities in myelocortical MS patients resulted from axonal changes, gliosis and disruptions in the blood-brain barrier in the absence of white matter demyelination. This hypothesis will be assessed by extensive immunohistochemical analysis of regions from myelocortical MS patients identified as being abnormal by MRI compared to regions considered normal on the myelocortical MS MRI as well as similar regions previously analyzed in classical MS patients from (47).

Finally, I hope to determine if there is a unique MRI metric or characteristic for myelocortical MS patients that would allow for their unique identification pre-mortem from the other MS subtypes. Additionally, I would want to answer the question if there would be differences in the amount of whole brain and white matter atrophy between our two MS cohorts?
Given that there are significantly fewer visible cerebral white matter lesions in the myelocortical MS patients, we hypothesized that MRI will differentiate myelocortical MS from classical MS by white matter lesion volumes and atrophy measures, particularly white matter atrophy. We expected the classical MS patients to have more white matter atrophy than the myelocortical MS patients. Investigation of this hypothesis requires the analysis of several MRI metrics including the number of MRI white matter lesions, total white matter lesion volumes, whole brain atrophy, white matter atrophy, gray matter atrophy, and cortical thickness, in order to determine differences between the myelocortical and classical MS patients.
Section 6.1 Patients and Methods

6.1.1 Subjects

We systematically reviewed all of the cases collected from the Cleveland Clinic MS rapid autopsy program and selected cases that fulfilled the following criteria: 1) diagnosis of MS during life, and 2) with 3 or less macroscopically visible white matter lesions on coronal slices from one hemisphere at time of autopsy (myelocortical MS). In this study, 12 MS patients met the previously stated criteria and are referred to as myelocortical MS, which were compared with 12 age- and EDSS-matched MS patients containing more than 3 macroscopic cerebral white matter lesions at time of autopsy (referred to as classical MS). The patients were chosen from a total of 97 MS cases. Two of the myelocortical MS patients were in the RRMS phase of the disease, 8 were in SPMS, and 2 were PPMS, whereas the classical MS cohort consisted of 8 patients in the SPMS phase and 4 PPMS patients (see clinical demographics in Table 1). There were 13 males and 11 females, with no difference between the two MS groups with respect to the mean age at death (68 years myelocortical MS, 63 years classical MS), disease duration (26 years, 28 years), brain weight (1177 grams, 1181 grams), postmortem time (8 hours, 6.1 hours) or disability measured by the expanded disability status scale (EDSS, medians 8.0, 8.0 respectively). Chart reviews of these patients revealed similar symptoms between the two groups, such as optic neuritis, transverse myelitis and cognitive changes were reported but no
formal cognitive testing was performed (Table 1). The majority of these patients were on disease modifying therapies over their disease course, however the myelocortical MS patients as compared to the classical MS patients, were frequently switched from one therapy to another, likely due to a lack of efficacy on their disease course (Table 2). All human tissue studies were approved by the Cleveland Clinic Institutional Review Board.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Myelocortical MS (N=12)</th>
<th>Classical MS (N=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS Type</td>
<td>2 RR, 8 SP, 2 PP</td>
<td>8 SP, 4 PP</td>
</tr>
<tr>
<td>Sex, % Male</td>
<td>50</td>
<td>58.3</td>
</tr>
<tr>
<td>Age at death, Median years (IQR)</td>
<td>71 (58.8-79.5)</td>
<td>61 (55.0-70.0)</td>
</tr>
<tr>
<td>Disease Duration, Median Years (IQR)</td>
<td>29 (13.0-33.5)</td>
<td>31 (22.3-36.0)</td>
</tr>
<tr>
<td>EDSS, Median (IQR)</td>
<td>8 (8-8)</td>
<td>8 (7.8-8.6)</td>
</tr>
<tr>
<td>Brain Weight, Median grams (IQR)</td>
<td>1180 (1067.5-1230.0)</td>
<td>1192.5 (1067.5-1285.0)</td>
</tr>
<tr>
<td>Postmortem Time, Median Hours (IQR)</td>
<td>7.93 (6.4-8.8)</td>
<td>5.42 (4.9-7.4)</td>
</tr>
<tr>
<td>Transverse Myelitis</td>
<td>6 Yes, 1 No, 5 NR</td>
<td>2 Yes, 1 No, 9 NR</td>
</tr>
<tr>
<td>Mental Decline</td>
<td>6 Yes, 6 NR</td>
<td>2 Yes, 10 NR</td>
</tr>
<tr>
<td>Paresthesia in Lower Extremities</td>
<td>6 Yes, 6 NR</td>
<td>12 NR</td>
</tr>
<tr>
<td>Optic Neuritis</td>
<td>2 Yes, 2 No, 8 NR</td>
<td>3 Yes, 9 NR</td>
</tr>
</tbody>
</table>

Table 1. Myelocortical and Classical MS patients’ clinical demographics are similar for age at death, disease duration, brain weight at death, and disability scores on the EDSS scale. There were no statistical differences for any of the above mentioned demographics between the two MS cohorts. Transverse myelitis was a frequent symptom in myelocortical MS, along with mental disturbances and sensory symptoms in the lower extremities. RR = Relapse-Remitting MS, SP = Secondary Progressive MS and PP = Primary Progressive MS IQR = Interquartile range NR = Not Recorded
### Disease Modifying Therapies

<table>
<thead>
<tr>
<th>Myelocortical MS Cases</th>
<th>Treatments</th>
<th>Classical MS Cases</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Solu-Medrol, Copaxone, Avonex, Rebif</td>
<td>1</td>
<td>Avonex, Steroids, Tysabri, Baclofen, Copaxone</td>
</tr>
<tr>
<td>2</td>
<td>Avonex, Natalizumab, Solu-medrol, Baclofen, Steroids</td>
<td>2</td>
<td>Cytoxan</td>
</tr>
<tr>
<td>3</td>
<td>Baclofen, Zanaflex, Copaxone, Steroids</td>
<td>3</td>
<td>NR</td>
</tr>
<tr>
<td>4</td>
<td>Baclofen</td>
<td>4</td>
<td>Steroids</td>
</tr>
<tr>
<td>5</td>
<td>Avonex, copaxone, Solu-medrol, Novantrone, Steroids, Baclofen, Neurontin</td>
<td>5</td>
<td>Cytoxan, Steroids</td>
</tr>
<tr>
<td>6</td>
<td>Avonex, Baclofen, Steroids, Cytoxan, Imuran</td>
<td>6</td>
<td>NR</td>
</tr>
<tr>
<td>7</td>
<td>NR</td>
<td>7</td>
<td>No DMT</td>
</tr>
<tr>
<td>8</td>
<td>Never on DMT</td>
<td>8</td>
<td>Methotrexate, Steroids</td>
</tr>
<tr>
<td>9</td>
<td>Solu-Medrol, Baclofen, Steroids</td>
<td>9</td>
<td>NR</td>
</tr>
<tr>
<td>10</td>
<td>Never on DMT</td>
<td>10</td>
<td>NR</td>
</tr>
<tr>
<td>11</td>
<td>Never on DMT</td>
<td>11</td>
<td>Methotrexate, Steroids</td>
</tr>
<tr>
<td>12</td>
<td>NR</td>
<td>12</td>
<td>NR</td>
</tr>
</tbody>
</table>

Table 2. The disease-modifying therapies (DMT) reported in the available patients’ records. It appears as though the myelocortical MS patients which received the disease modifying therapies were not effective, since many were switched multiple times from one therapy to another, more so than in the classical MS patients. Steroids are commonly given to patients at the time of a relapse to reduce the severity of the symptoms.
6.1.2 Postmortem Tissue Processing

All donors had consented to tissue donation prior to their death or next of kin consented at the time of the patients’ death. Following a postmortem MRI scan, human brains and spinal cords were removed from deceased MS tissue donors. Brains were weighed and one cerebral hemisphere was immediately separated from the rest of the brain tissue and fixed intact in 4% paraformaldehyde for at least one month (long fixation) before being scanned ex vivo and sliced in a coronal orientation at a thickness of 1cm and photographed for MRI-histopathological correlative studies. The remaining hemisphere was immediately cut into 1 cm-thick coronal slices with the odd numbered slices being fixed in 4% paraformaldehyde for 2-5 days, while even numbered slices were photographed on the anterior and posterior views and then snap-frozen using a dry ice and isopentane slurry. The motor cortex was inked on both of the intact hemispheres prior to slicing for identification on the coronal brain slices. The brainstem was dissected apart from the cerebellum, then fixed in 4% paraformaldehyde en bloc for 2-5 days and later sectioned every 4 mm in the horizontal plane for gross examination of demyelination. Right and left cerebellum were separated and then each were sliced in the horizontal plane into four equal sections, with the right sections being frozen and the left being fixed in 4% paraformaldehyde for 2-5 days. Spinal dura was removed and examined for dorsal root ganglia, which were fixed or frozen. Nerve roots were separated into anterior and posterior for both the left and right sides. All left nerve roots were fixed in 4% paraformaldehyde for 2-5 days, while the right nerve roots were frozen. The spinal cord was examined grossly for lesions and then cut into 2cm sections beginning at the sacral end of the cord, with alternate segments fixed in 4% paraformaldehyde or frozen. The rostral end of each spinal cord segment was inked for identification later. All tissue was photographed on both anterior and posterior sides for
identification of macroscopic demyelinating white matter lesions. Regions of interest for histological analysis were excised, cryoprotected, and sectioned at 30 µm thickness using a freezing-sliding microtome.

6.1.3 Quantification of Macroscopic Cerebral White Matter Lesions

The total number of cerebral macroscopic white matter lesions were counted on all the coronal slices (roughly 12 per case) from the long-fixed hemisphere for all the MS brains included in this study. The number and area occupied by cerebral white matter demyelination for each MS case was calculated from the photographs in Adobe Photoshop by taking the number of pixels in the white matter lesion and then dividing by the number of pixels in a 1 cm² region based on a scale bar included in the photographed image of the slice, resulting in the area of the lesion in centimeters squared. This was performed on the anterior and posterior images for every slice with a white matter lesion on one hemispheric side (short fixed side). Lesions that were counted and measured on the posterior view of a proceeding slice were not included in the analysis of the anterior view on the following slice. The criteria for including any of the white matter discolorations as a demyelinating cerebral white matter lesion required a minimum area of 0.3 cm or greater, since anything smaller in area could be due to vascular induced changes and not demyelination. This is also the inclusion criteria for an MRI lesion. Cerebral white matter lesions for each of the cases were determined by two investigators (one of which was a trained neuropathologist), and any disagreements were examined together, discussed and agreed upon.
The sum of all the cerebral white matter lesions areas for one case was calculated and this number was compared between the two MS groups.

6.1.4 Region Selection

For all MS cases, cerebral tissue blocks containing five predetermined neocortical regions (cingulate, superior frontal, superior temporal, motor, and insular cortices, Fig. 3A), the hippocampus, thalamus, and four segments of different spinal cord levels (1 cervical, 2 thoracic and 1 lumbar) were cryoprotected, frozen and sectioned at 30µm on a freezing-sliding microtome for histological analysis. Segments of spinal cord with macroscopic lesions on their superficial surface were preferentially sampled from both MS cohorts; however, if no lesions were grossly visible then the lumbar section was sampled approximately 4 cm from the conus, the thoracic sections were taken approximately 12 cm and 24 cm from the conus and the cervical section was approximately 36 cm from the conus. Brainstem was examined for the presence of macroscopic lesions in the 4 mm thick slices.

6.1.5 MRI Procedures

Tissue sampling in brain white matter was based on analysis of postmortem MRI scans, with regions of interest defined in a similar manner as in our previous MRI-pathology correlation studies (47). Briefly, brains were imaged \textit{in situ} immediately prior to autopsy using a
standardized postmortem MRI acquisition consisted of a T2-weighted fluid-attenuated inversion recovery (FLAIR) image, a T1-weighted spin echo image, a pair of proton density-weighted images to calculate magnetization transfer ratio, and a 3D 1mm isotropic T1-weighted magnetization prepared rapid gradient echo image (MPRAGE) for co-registration between the different scan images. The FLAIR images were analyzed to segment T2-hyperintense regions, which were then further characterized based on T1 and MTR characteristics. Following tissue fixation, the long-fixed hemisphere from each brain was re-imaged just prior to slicing in a custom-made box with MRI-visible markers demarcating each slice plane. The post-mortem images were then co-registered with the post-fixation images and reformatted in order to obtain maps of MRI characteristics corresponding to each fixed tissue slice (Fig. 2). Finally, for each brain, four types of MRI-defined regions-of-interest were selected for histological analysis: normal-appearing white matter, T2-only (abnormal signal on FLAIR only) white matter, T2T1MTR (abnormal on all 3 image types) white matter, and heterogeneous (abnormal signal with one portion T2-only and another portion T2T1MTR) white matter and are discussed in the following chapter. Histological analysis was performed blinded to the MRI categorization of each tissue region of interest. These MRI-defined white matter regions of interest for histological analysis were excised, cryoprotected, and sectioned at 30 µm thickness using a freezing-sliding microtome for immunohistochemical analysis.

MRIs were analyzed to calculate and compare brain parenchymal fraction (BPF), white matter fraction, gray matter fraction and cortical thickness in myelocortical and classical MS cohorts to identify any specific differences in tissue loss between the two groups. The whole brain is segmented and BPF is calculated from the acquired FLAIR images as described previously (104, 220). Whole brain atrophy was measured by BPF, which provides a
reproducible estimation of brain volume irrespective of head size. GM and WM volumes were measured using an automated probability-based tissue segmentation algorithm that incorporates intensity, anatomical, and morphological information (221). Cortical thickness was assessed as described previously (222). We do not perform postmortem MRIs on control brains, therefore no comparison to this patient population can be made. Classical and myelocortical MS tissue volumes were compared with normal brain volume measures obtained from historical data obtained from living control patients. Control, classical and myelocortical MS brains were be matched for chronological age.

**Methods of MRI White Matter Region Selection**

Figure 2. Regions of interest are identified by MRI and tissue image coregistration. The ROIs are mapped and then exercised from the tissue for histochemical analysis.
6.1.6 Immunohistochemical Analysis

Free-floating sections were microwaved in 10 mM citric acid buffer (pH 6.0) for 5 minutes and incubated for 30 minutes in phosphate-buffered saline with 3% hydrogen peroxide and 10% Triton X-100. The immunostaining was performed by the avidin-biotin-complex method using diaminobenzidine as the chromogen as previously described (48). The following primary antibodies were used: rat anti-proteolipid protein (PLP, gift from Wendy Macklin), mouse anti-Major-Histocompatibility-Complex Class II (CR3/43, Dako, Glostrup, Denmark), rabbit anti-glial fibrillary acidic protein (GFAP, Z0334, Dako, Glostrup, Denmark), mouse anti-HuR (1:500, 19F12, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-synaptophysin (1:1000, clone SY38, Dako, Glostrup, Denmark), mouse anti-Map2 (1:1000, AB5622, Millipore, Temecula, CA), mouse anti-SMI-31 (1:1000, 14944502, Covance, Emeryville, CA), mouse anti-SMI-32 (1:1000, E10167EF, Covance, Emeryville, CA), rabbit anti-fibrinogen (F0111, Dako, Glostrup, Denmark), rabbit anti-albumin (F0117, Dako, Glostrup, Denmark), goat anti-human IgG (Jackson ImmunoResearch), mouse anti-CD3 (F7.2.38, Dako, Glostrup, Denmark), and mouse anti-CD20 (L26, Dako, Glostrup, Denmark). Tissue was incubated in primary antibodies for two days at 4°C and secondary antibodies for one hour at room temperature at a concentration of 1:500. The secondary antibodies utilized were biotinylated goat anti-rat, anti-rabbit or anti-mouse IgG (Vector Laboratories, Burlingame, CA).
6.1.7 Tissue Processing and Paraffin Embedding of Hemispheric Sections

The cerebral hemisphere that was fixed for one month was rescanned ex vivo by a 3Tesla MRI scanner in a specialized brain slicing box with MRI detectable markers along the top edge of the box, and sliced into 1 cm-thick coronal sections along the slots in the boxes aligned with the MRI detectable markers. The coronal brain slice (usually slice 7, 8 or 9) at approximately the level anatomically containing the posterior portion of the hippocampus, thalamus, insular cortex and the posterior end of the putamen was selected for tissue processing for paraffin embedding and hemispheric sectioning from each of the available cases in this study. The hemispheric slices were first rinsed from the cryoprotection with 0.04% Sorenson’s Buffer and then placed in 50% dehydrant ethanol based solution for at least 12 hours, followed by 70% dehydrant for 24-48 hours and then placed in a VIP3000 Tissue Processor which is set to a 23 hour program consisting of the following: a half hour in 70% dehydrant, 1 hour in 80% dehydrant, 3 hours in 95% dehydrant, 10 hours in 100% dehydrant, 5 hours in xylene and 5 and a half hours in infiltrating paraffin under a vacuum seal. After the tissue is processed, the hemispheric brain slices were embedded in embedding paraffin, set aside in a cold water bath to cool and then sectioned at a thickness of 7 µm on a sledge microtome and immunostained with antibodies for myelin (proteolipid protein), microglia (MHC class II), immune cells (CD45), T cells (CD3) and B cells (CD20), as well as a cresyl violet stain for cell nuclei. The density of only CD45+ cells were quantified in subpial cortical lesions and associated meninges; CD3+ and CD20+ were not quantified due to the very few cells observed with the general CD45 stain.
6.1.8 Quantification of Histology

All slides of tissue blocks stained for PLP were scanned using a slide scanner (PathScan Enabler IV, Meyer Instruments, Houston, TX). Lesions were defined as areas negative for myelin staining. ImageJ (NIH, http://rsweb.nih.gov/ij) was used to measure and calculate lesion load, which was defined as the lesion area divided by the total area of the tissue being examined. Gray matter lesion load was quantified for neocortical and thalamic sections (Fig. 3B). Gray matter and white matter lesion loads were calculated separately for hippocampal and spinal cord sections (Fig. 3C). In the spinal cord, lesion areas were also measured using ImageJ and then averaged among all spinal cord levels.
Methods of Lesion Load Measurements

Figure 3. Five cortical regions were excised from the coronal brain tissue slice and included the cingulate gyrus (A1), the superior frontal gyrus (A2), motor cortex (A3), superior temporal gyrus (A4), and the insular cortex (A5). Subpial lesion loads were measured by outlining the lesion (black line) and then dividing by the entire cortical area outlined (red line, B). Spinal cord lesion loads were segregated into white matter and gray matter lesions (black lines) and divided by the total white matter (red line) or gray matter (yellow line), respectively (C).

The MRI white matter regions of interest containing myelin loss were measured and the area of myelin loss was divided by the total region of interest area to identify the percentage of the region of interest that was demyelinated. In order to analyze whether there were differences in the amount of myelin present at a microscopic level in each of the MRI regions of interest, myelin density measurements were calculated by taking 4 non-overlapping micrographs with a Zeiss Microscope and Magnafire CCD Camera of the PLP stained slides within the MRI white matter region of interest at a magnification of 40x. These micrographs were then grayscaled
followed by thresholding for the myelin stain using ImageJ, after which the density of the area was measured and recorded in Microsoft Excel. The four densities were then averaged and the mean densities for all MRI-defined normal appearing white matter were compared against the mean densities for the T2T1MTR regions. Similar procedures were used to measure the axonal densities based on SMI31 and SMI32 staining in these same MRI defined regions. Axonal counts from the same images used for density measurements were performed using the particle counter which was set to 4 pixels or higher as previously described by Bjartmar and colleagues (54). The average of the 4 micrographs for each region was calculated and compared between the normal appearing white matter and T2T1MTR groups. Finally, the axonal diameter was calculated by dividing the average axonal density by the average axonal count for the same region of interest.

Activated microglia were identified by the marker MHC Class II which labels only activated microglia and macrophages. They were also identified morphologically by short, thick processes retracting to the cell bodies, unlike their resting state with small cell bodies and long, thin processes extending out into the brain parenchyma. Activated microglia were examined for any patterns within the tissue across all cases. In the MRI-defined regions of interest, 3 micrographs of the class II stained microglia were taken, followed by a threshold analysis on the micrographs in ImageJ for the microglia and the density of these cells was measured and compared between the different MRI categories.

To assess the loss of neurons, nuclei were stained with HuR and examined on a light microscope for cell loss within subpial lesions. I examined synaptophysin staining by light microscopy for possible changes in synapses within subpial lesions. I also examined Map2 staining by light microscopy for possible morphological changes in dendrites within subpial
lesions. Lastly, to assess the amount of inflammatory infiltration within and around subpial lesions, we stained with CD20 (B-cells), CD3 (T-cells), and leukocyte common antigen (LCA/CD45, stains all inflammatory cells). Hemispheric slices adjacent to PLP stained slices were stained with LCA and meninges adjacent to regions of subpial demyelination on the PLP stained slices were measured (length in μm) and the LCA positive cells were counted. The total number of cells was divided by the total meningeal length to assess the association of meningeal inflammation with subpial demyelination.

Section 6.2 Research Design

The myelocortical MS patients are a unique cohort of MS patients not described previously; therefore, this thesis discusses the comparison of the myelocortical MS patients’ pathology, and in particular, demyelination in various regions throughout the central nervous system, to the pathology in the same regions of age- and disability- matched classical MS patients. This work was performed to not only characterize the myelocortical MS patients, but to investigate the presence of cortical demyelination independent of cerebral white matter lesions. In addition, MRI metrics were analyzed for possible differences in MRI abnormality volumes, whole brain and white matter, gray matter, and cortical atrophy measurements, which would possibly identify the myelocortical MS patients from the classical MS patients pre-mortem and may provide some insights into the disease pathogenesis of MS. Furthermore, examination of what pathologies resulted in the abnormal MRI signal in the cerebral white matter of myelocortical MS patients were examined, since cerebral white matter demyelination was not a major feature macroscopically. These results will be compared to the results of a similar study.
performed in classical MS patients in a paper by Fisher and colleagues to better extrapolate what pathologies can result in a white matter lesion by MRI (47).

Section 6.3 Data Analysis and Statistics

6.3.1 Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, Inc., La Jolla, CA). Fisher’s exact test was used for categorical data. Data are expressed as mean ± standard error of the mean and Student’s $t$ tests (paired or unpaired) were used for some comparisons. If a data set failed normality tests, Mann-Whitney U test was used when appropriate. When multiple comparisons were performed, Bonferroni’s correction was applied. Some analyses required two-way or three-way ANOVAs with the Holm-Sidak method for multiple comparison correction. Groups were considered significantly different at $p< 0.05$. 

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Chapter 4

Section 7.1 Results

7.1.1 Characterization of spinal cord, cortical and deep gray matter lesions in the absence of white matter lesions

In a collection of 97 postmortem MS brains, we identified 12 MS patients containing less than 3 macroscopic cerebral white matter lesions in 1-centimeter-thick coronal brain slices at the time of autopsy, and were referred to this cohort as myelocortical MS (MCMS). The prevalence for this subtype of MS is roughly 12% in our collection, suggesting that this subtype could be as frequent as primary progressive MS in the MS population. The purpose of these initial studies was to characterize the regions of demyelination in cerebral gray matter and spinal cord of the myelocortical MS patients essentially devoid of white matter lesions, which resulted in the accumulation of severe disability at time of death (mean 7.7 on the Expanded Disability Status Scale, EDSS) and to determine the correlation between that cerebral white matter demyelination and cortical demyelination. The extent of demyelination within these 12 myelocortical MS cases was compared to 12 classical MS (CMS) patients that at the time of autopsy contained several macroscopic cerebral white matter lesions and were age- and EDSS-matched to the myelocortical MS cases (see Table 1 for Patient Demographics). All patients included in this study had a clinical diagnosis of MS. There were no differences between the myelocortical and classical MS patients with respect to disease duration (means 28 and 26 years respectively), MS
type, brain weight (1177 and 1180 grams) or postmortem time (8 and 6.1 hours). The major difference between these two groups was the presence of macroscopic cerebral white matter lesions at the time of autopsy.

The number of macroscopic cerebral white matter lesions and area affected by cerebral white matter demyelination were quantified from 1 cm coronal brain slices of one hemisphere from both the classical (Fig. 4A) and myelocortical MS patients (Fig. 4B). This analysis was performed to determine how much of a difference in the amount of cerebral white matter demyelination there was between the two MS cohorts. Discolorations of the white matter were considered to be demyelinating lesions if they were greater than 3mm$^2$, since anything smaller many be due to vascular changes. The number of white matter lesions was significantly greater in the classical MS patients (an average of 12 lesions per hemisphere) compared to the myelocortical MS patients (0.5 lesions per hemisphere, $p < 0.0001$, Fig. 4C). The area occupied by white matter lesions was then quantified and further demonstrated drastic differences, with the classical MS patients having on average a little over 12 cm$^2$ affected by white matter demyelination and only an average 0.24 cm$^2$ was demyelinated in the myelocortical MS patients ($p < 0.0001$, Fig. 4D). So not only did the myelocortical MS patients have fewer numbers of white matter lesions but the lesions were also 60 times smaller in size, confirming the general lack of cerebral white matter demyelination in the myelocortical MS patients. The myelocortical MS patients had very little white matter demyelination, but accumulated severe disability, therefore other regions of the central nervous system must have been demyelinated and resulted in this disability. The EDSS score is highly weighted on ambulation and therefore is greatly affected by spinal cord demyelination, making this an obvious first region to examine.
Cerebral White Matter Lesion Loads

Figure 4. Classical MS patients have several macroscopic white matter lesions visible on coronal hemispheric sections (A). Myelocortical MS patients, in contrast, have very few to no macroscopic white matter lesions visible in their coronal hemispheric sections (B). This drastic difference between the two MS groups is illustrated by the significantly greater number of white matter lesions in one hemisphere of the classical MS brains as compared to the one brain hemisphere of the myelocortical MS (C). This is further demonstrated by the significantly greater area occupied by white matter lesions in the classical MS patients compared to myelocortical MS patients (D).

7.1.1.1 Characterization of spinal cord demyelination in classical and myelocortical MS patients

It has been previously shown that spinal cord lesions correlate better with MS patients’ disability scores than the cerebral white matter lesion load (283). Spinal cord demyelination was abundant in both the gray and white matter regions of the spinal cord for both the classical (Fig. 5A) and myelocortical MS patients (Fig. 5B). The demyelinating lesions within the spinal cord seldom respected the white/gray matter border, as only rarely were lesions restricted to the white matter (7 lesions) or gray matter (3 lesions) between the two MS groups which had a total of 53 spinal cord lesions. The average number of spinal cord segments with demyelination was no
different between the two MS groups (Fig. 5C), suggesting that this region of the central nervous system was equally vulnerable to the incidence of demyelination, with an average of 2.7 segments containing demyelination in the classical MS cases and 2.3 segments in the myelocortical MS cases (p = 0.37). The spinal cord lesions were enough for a pathological confirmation of the MS diagnosis for 11 of the classical and 10 of the myelocortical MS patients. Despite the similarity in numbers of lesions, there was a difference in the percent area occupied by demyelination when quantified for the gray matter and white matter regions separately, with the classical MS spinal cords having greater amounts of demyelination (50.3% and 29.6% in gray and white matter, respectively), as compared to the myelocortical MS spinal cords (30.3% and 13.3% respectively, Fig. 5D). The classical MS patients had significantly higher percentage of gray matter demyelination as compared to the percentage of white matter demyelination (p = 0.006), this same trend was observed in the myelocortical MS patient cohort, but it did not reach statistical significance (p = 0.25). Although the classical and myelocortical MS patients contained the same number of spinal cord lesions, the classical MS lesions were slightly larger in size in both the gray matter (p = 0.017) and the white matter (p = 0.008) compared to those present in the myelocortical MS spinal cords. There was no difference in the ratio of gray matter to white matter demyelination between the classical and myelocortical MS spinal cords. Furthermore, when the gray matter area, white matter area and total area were measured for all segments of the spinal cord, there was no differences between these two MS cohorts (p = 0.23, p = 0.66, and p = 0.38, respectively), suggesting that spinal cord atrophy was also likely to be similar between the two MS cohorts. This demyelination within the spinal cords of the myelocortical MS patients was likely a major contributor to the disability which accumulated in these patients. The cognitive impairment recorded for several of the myelocortical MS patients
in this study would be due to demyelination elsewhere, therefore, other regions of the central nervous system were investigated for demyelination in the absence of the cerebral white matter lesions in these unique myelocortical MS patients.

Figure 5. Both gray matter and white matter demyelination developed in the classical MS spinal cord, irrespective of the boundaries between these two tissue types (A). Similarly, myelocortical MS patients’ spinal cords also displayed gray and white matter demyelination (B). There was no difference in the number of spinal cord segments that contained a lesion between the classical and myelocortical MS cohorts (C). The percent of demyelinated area was significantly greater for both the gray and white matter demyelination in the classical MS spinal cords compared to myelocortical MS (D). There was also a significant difference in the amount of demyelination between the gray and white matter of the classical MS spinal cord (D). Scale bars = 200µm.
7.1.1.2 Characterization of cortical demyelination in classical and myelocortical MS patients

For these next studies, 5 predetermined cortical regions were examined due to their high incidence of subpial demyelination (cingulate gyrus, insular cortex, superior frontal gyrus, superior temporal gyrus, and motor cortex). In some cases, the role of demyelinating lesions in contributing to disability scores is easily understood, such as in the motor cortex which initiates and coordinates complex voluntary movement, other cases, it is less straightforward, as in the insular cortex, which plays a role in cardiovascular and autonomic functions. Demyelination of the cerebral cortex comes in three distinct patterns of lesions, the first being leukocortical lesions which are thought to begin in the subcortical white matter and expand up into the deeper layers of the cortex. The second type of cortical lesion is the intracortical lesion, which are small pockets of demyelination present strictly within the cortex and usually surrounding a blood vessel. The last type of cortical lesion is the subpial lesion, and these lesions begin at the pial surface of the brain, continue down usually into layers 3 or 4 of the cortex and can extend across multiple gyri. The frequency of these three types of cortical lesions was surveyed in both the MS cohorts within the 5 cortical regions. Leukocortical lesions were observed in 8 of the 12 classical MS cases, with an average of 1 per case within the 5 cortical regions examined. In contrast, leukocortical lesions were not detected in myelocortical MS cases. The paucity of leukocortical lesions in the myelocortical MS patients is evidence of leukocortical lesions beginning in the white matter and extending into the cortex, since patients with no cerebral white matter demyelination lack this type of lesion. There were no differences in the number of intracortical lesions between the classical MS which had an average of 3 lesions per case, compared to the 2 lesions per case in the myelocortical MS cases (p = 0.24). However,
intracortical lesions are small and probably do not contribute much to the disability of MS patients. Also some of the intracortical lesions may not be demyelination, but rather could be vascular abnormalities.

Since subpial demyelination is the most common and extensive of all the cortical lesions, and is the most likely of the cortical lesion types to contribute to the overall accumulated disability in MS patients, the extent and frequency of subpial demyelination was quantified within the 5 cortical regions. The overall frequency of subpial demyelination was slightly greater in the classical MS patients having on average 3 cortical regions with subpial demyelination compared to 1 on average in the myelocortical MS patients (p =0.014). This ratio of 3 to 1 a more modest difference in pathology when compared to the 69 to 1 ratio of the cerebral white matter lesions. The frequencies of subpial demyelination varied between the different cortical regions as well, with the superior temporal gyrus being the most frequently demyelinated, followed by the cingulate gyrus, insular cortex, superior frontal gyrus and finally the motor cortex. Even though the number of subpial lesions was increased in the classical MS patients the lesions themselves were of similar size as determined by the percent area of demyelinated cortex with the myelocortical MS having a slightly larger area affected with an average of 29.5% of the cortex, compared to the 23.5% cortical area demyelinated in the classical MS patients (p = 0.21). When total area occupied by subpial demyelination was measured from the hemispheric sections stained with PLP there was a greater amount of subpial demyelination in the classical MS patients (13.4%) compared to the myelocortical MS patients (4.4%, p =0.09). Although there were more lesions observed in the classical MS patients, when the subpial lesions occurred they were of the same size as those observed in the myelocortical MS patients. Subpial demyelination in both cohorts affected layers 1-4 of the cortex and often
extended over multiple gyri as previously described (236). Furthermore, no differences were apparent for changes in the staining of astrocytes or patterns of activated microglia within the regions of subpial demyelination between the classical and myelocortical MS lesions.

Cortical Gray Matter Lesion Loads

Figure 6. Subpial demyelination is extensive in both the classical MS cortical tissue blocks (A) as well as the myelocortical MS cortical tissue blocks (B). The number of cortical regions containing subpial demyelination was significantly greater in the classical MS than in the myelocortical MS patients at a ratio of 3 to 1 (C). However, when lesions were present in both cohorts the lesions occupied roughly the same percent area (D), making all the subpial lesions regardless of MS type the same size. Scale bars = 200µm.
Each of the MS cases were examined for demyelination in a complete coronal slice from the long fixed hemisphere containing the same 5 cortical regions examined in the tissue blocks, the hippocampus, and the thalamus. The coronal hemispheric brain slices were paraffin-embedded, cut into 7µm thick sections, and stained with antibodies against proteolipid protein. The hemispheric sections were examined from 9 of the classical MS cases and 8 of the myelocortical MS cases, which confirmed the findings from the tissue blocks. White matter lesions and leukocortical lesions were only observed in the classical MS patients (Figure 7A, blue and yellow areas respectively), and subpial demyelination was observed in both the classical and myelocortical MS coronal slices (red regions on Figure 7A and 7B respectively).

**Coronal Images of Classical and Myelocortical Multiple Sclerosis**

Figure 7. Hemispheric section stained with proteolipid protein from a classical MS case demonstrates the extensive white matter demyelination (blue), leukocortical lesions (yellow) and subpial demyelination (red, A). A hemispheric section from a myelocortical MS case contains only subpial demyelination (red) but lacks white matter and leukocortical lesions (B).
7.1.1.3 Characterization of demyelination and association with meningeal inflammation on hemispheric sections

These 7µm coronal hemispheric sections were also utilized to analyze the association between subpial demyelination and the presence of meningeal inflammation. Therefore, adjacent tissue sections to those stained with PLP for myelin were stained for LCA-, CD20- and CD3-positive cells, which would demonstrate the presence of peripheral immune cells within the intact meninges. A hemispheric section from a B-cell Lymphoma patient stained for LCA (CD45), the pan-lymphocyte marker, demonstrated widespread infiltration of lymphocytes into the brain parenchyma (Fig. 8A). The same staining in the MS cases showed very few lymphocytes were present in the brain or meninges associated with subpial demyelination (Fig. 8B). When the number of lymphocytes were counted and divided by length of intact meninges, both the classical and myelocortical MS patients had an average of 0.004 and 0.003 cells per µm of meninges, respectively (Fig. 8C). Similar to the LCA staining pattern, the hemispheric sections stained with CD3 (T cell marker) and CD20 (B cell marker) in the lymphoma brain had widespread brain parenchymal infiltration (Fig. 8D), whereas the MS cases rarely contained positive staining for T or B cells (Fig. 8E). It is estimated that 1 out of every 50 to 100 LCA positive cells is a B cell, so cells positive for CD20 were also counted in the meninges of the MS cases. The B cell counts were no different between the classical and myelocortical MS patients and were a very small proportion of the LCA positive cells in the meninges (Fig. 8F). Therefore, since so few cells were present in the intact meninges along the brain surface and in the deep sulci, it was concluded that B-cell follicle like structures are not formed in these MS cases and furthermore do not play a role in the mechanism of subpial demyelination in these MS cohorts.
Peripheral Inflammatory Cell Counts

Figure 8. Several hundreds of lymphocytes invade the brain parenchyma in a Lymphoma case (A), however no lymphocytes are found in the parenchyma and very are few present in the meninges of the MS cases (B). The number of LCA (CD45+) cells in the meninges was very low and not different between the two MS groups (C). B cells infiltrate the brain of the Lymphoma case (D), but B cells are rarely observed in the MS cases like the cell in the blood vessel of this MS case (E). The number of B cells in the meninges was almost nonexistent and no difference was detected between the MS groups (F).

7.1.1.4 Characterization of hippocampal lesions in classical and myelocortical MS patients

The hippocampus is a unique structure of the brain in which the gray matter and white matter are intimately intertwined together. This region of the brain is frequently demyelinated in MS patients and likely contributes to the cognitive deficits observed in 40-60% of MS patients due to its function in learning and memory. Hippocampal demyelination results in neuronal
changes, particularly in the levels of proteins for pathways of axonal transport, synaptic plasticity, glutamate homeostasis, learning and memory, and neuronal survival. These studies suggest that demyelination greatly impacts the vulnerability and viability of neurons. As previously mentioned, some of the myelocortical MS patients had developed cognitive impairment, which suggests hippocampal demyelination may have occurred during their disease course. Therefore, to determine whether hippocampal demyelination contributed to the cognitive deterioration in these patients, one hippocampus from each of the myelocortical and classical MS patients was examined for the presence of demyelination within the gray matter and white matter. Eleven of the twelve classical MS cases had hippocampal demyelination which affected both the gray matter as well as the white matter tracts (Fig. 9A). Ten of the myelocortical MS patients had a more interesting pattern of demyelination, with the gray matter being greatly affected, but a relative sparing of the hippocampal white matter tracts (Fig. 9B). It is possible that the 3 MS cases that did not display hippocampal demyelination could have had demyelination in an anterior region of the hippocampus, or that the hippocampus on the opposite hemisphere of the brain could have been demyelinated. The white matter tracts, strata lacunosum and radiatum of the hippocampus (which contain the Schaeffer collaterals of CA3) were relatively spared from demyelination in all but one of the MCMS brains; while the alveus (consists of subventricular WM projection fibers) was spared in nine of the myelocortical MS cases, with varying degrees of demyelination in the other four cases. There was no difference between the classical and myelocortical MS with respect to demyelination of the hippocampal gray matter (31% and 30% respectively, p = 0.28), but there was significantly more demyelination of the hippocampal white matter in the classical MS patients (22%) compared to the myelocortical MS patients (6%, p = 0.043, Fig. 9C). The myelocortical MS hippocampal gray matter demyelination was
significantly greater than white matter demyelination ($p = 0.002$), which further demonstrates the
gray matter dominant demyelination of this MS subtype (Fig. 9C). The pattern of demyelination
of the hippocampus mimics the pattern observed in the rest of the cerebrum, with the
myelocortical and classical MS patients have relatively similar amounts of gray matter
demyelination but significantly less demyelination in the myelocortical MS patients’ white
matter.

7.1.1.5 Characterization of thalamic lesions in classical and myelocortical MS

The thalamus is a deep gray matter structure that has been shown to be affected by
degeneration resulting in thalamic atrophy early in MS. It is not, however, a common location
for MS demyelinating lesions. The prevalence of spinal cord and cortical gray matter
demyelination within the myelocortical MS patients led to the hypothesis that there would be
more thalamic demyelination in the myelocortical MS patients compared to the classical MS
patients due to the predominant gray matter demyelination of the myelocortical MS patients.
Similar to what has been previously shown for thalamic demyelination (55,81,82), thalamic
lesions were rare in both the classical (6 cases) and myelocortical MS patients (3 cases). These
thalamic lesions were small in size only taking up a few millimeters of space in the thalamus, but
were often surrounding a blood vessel with extensive microglial activation in and around the
lesion.
Figure 9. Hippocampal demyelination was observed in a majority of the classical and myelocortical MS patients, but the classical MS patients’ hippocampi contained demyelination of both the gray and white matter (A) in contrast to the myelocortical MS patients’ hippocampi which had predominantly gray matter demyelination (B). This observation was quantified by the percent demyelinated area of the gray or white matter of the two MS cohorts. There was significantly more gray matter demyelination than white matter demyelination within the myelocortical MS patients, in addition to greater amounts of white matter demyelination in the classical MS hippocampi compared with the myelocortical MS hippocampi (C). Scale bars = 200µm.

7.1.1.6 Identification of lesions in the brainstems of classical and myelocortical MS patients

Lesions within the brainstem can have effects on many functions including sensory or motor functions, since tracts from the spinal cord transverse this region on their way to the
thalamus and eventually the cortex. Also, the breathing centers of the midbrain can be affected by demyelinating lesions, which can result in death of the patient. Demyelination of the brainstem was examined grossly on 4mm thick cross-sections of all the patients in this study. Although some lesions were visible prior to slicing of the brainstem, upon sectioning several more demyelinated lesions became visible macroscopically. The number of visible lesions was counted for both of the MS cohorts. There were greater amounts of macroscopic lesions with the brainstems of the classical MS patients, 6 lesions on average compared to the myelocortical MS patients, which contained a mean of 2 lesions with their brainstem. However, the lesions appeared slightly bigger in size in the myelocortical MS cases, but this was not quantified.

7.1.1.7 Summary of histological examination of CNS of classical and myelocortical MS patients

The overall summary of the gross and histological examination of the central nervous system from the myelocortical MS patients, demonstrated a new pathological MS variant in which the regions greatly affected by demyelination were the spinal cord and cerebral cortex, although demyelination was also present in the hippocampus and brainstem in many of these patients. In comparison to the extent of demyelination present in the classical MS patients, two possible concepts were entertained, the first being that similar amounts of spinal cord and cortical demyelination may have resulted due to a similar mechanism of demyelination occurring in these regions between the two MS groups, and the second being that with such vast differences in the amount of cerebral white matter demyelination, there is likely a difference in the mechanism of demyelination within this region in which the classical MS probably have a
greater immune mediated mechanism which is not present in the myelocortical MS cases. This premise is discussed further in Chapter 5.

7.1.2 Pathology underlying magnetic resonance imaging abnormal signals in myelocortical MS patients

In order to understand the underlying pathology resulting in the white matter changes on MRI scans, an MRI and histopathological study was performed. The analysis of MRI changes in the white matter was implemented in 10 myelocortical MS cases that had postmortem MRI scans completed at the time of autopsy. MRI T1-weighted scans, T2-weighted scans, and MTR scans were examined for the presence of white matter abnormalities. First, MRI-defined regions of similar size were selected and the corresponding tissue was excised and analyzed blinded to the MRI category. The MRI defined white matter regions of interest fell into four categories: those regions which were normal appearing white matter on all of the MRI modalities; the T2 only regions which contained hyperintensities of white matter on the T2-weighted images, but appeared normal on the other two MRI modalities; the T2T1MTR regions which were hyperintense on the T2-weighted scan, and the MTR scan, but hypointense on the T1-weighted scan; and lastly the heterogeneous regions where lesions had parts which were T2 only and parts of the lesion were abnormal on T2T1MTR scans. The MRI white matter lesions which were abnormal on all the modalities (commonly called black holes) are thought to be more severe pathologically (such as more axonal damage and loss) and are probably more chronic than the white matter regions abnormal only on the T2-weighted scans. However, Fisher and colleagues
demonstrated in classical MS patients that 45% of the MRI defined white matter lesions on T2-weighted scans contained normal amounts of myelin and 17% of the MRI defined white matter lesions on T2-, T1-weighted scans, and low MTR values are not demyelinated. This led to the authors concluding that other MS related pathologies can result in the changes observed in white matter by MRI (47).

7.1.2.1 MRI –defined normal appearing white matter in myelocortical MS

The MRI defined normal appearing white matter and white matter lesions in the myelocortical MS cases were investigated for the presence of demyelination, axonal pathology, microglial activation, astrogliosis and breakdown of the blood-brain barrier. In the 46 regions for which the MRI defined as normal appearing white matter, similar to what was previously shown in classical MS patients, all of these regions had normal myelin throughout the entire region of interest (Table 3, Fig. 10A). There was minimal microglial activation observed by Class II in 37 of the 46 regions, (Fig. 10B) which could have been due to other mechanisms, such as the condition prior to death or the general cause of death. The area occupied by Class II staining, a marker of activated microglia was on average only 7% of the region of interest, providing further evidence of the minimal activation in these areas. There was very little presence of astrogliosis (7 of the 46 regions) and no evidence of breakdown of the blood-brain barrier as demonstrated by the lack of serum proteins staining in the brain parenchyma (Fig. 10C). These data suggest two observations, the first being that the normal appearing white matter in the myelocortical MS patients has a similar myelin status to classical MS normal
appearing white matter, absence of gliosis and intact blood-brain barrier. Secondly, the lack of pathology in these MRI-defined normal appearing white matter regions of interest allow for easier interpretation of the pathologies present in the MRI-defined white matter lesions and their influence on MRI signal changes.

7.1.2.2 MRI-defined T2-only regions in myelocortical MS

The 33 MRI white matter changes observed only on the T2-weighted MRI scans from the myelocortical MS patients were not demyelinated except for one, in which the demyelinated area only affected a small percentage (0.5%) of the MRI defined region of interest (Table 3, Fig. 10D). These data further confirm the macroscopic observation of a lack of cerebral white matter demyelination of the myelocortical MS patients. All of these T2-only regions of interest had microglial activation within the MRI defined lesion (Fig. 10E), and the area occupied by staining for the activated microglia marker Class II (10.1%), was significantly greater than the same density measured in the normal appearing white matter MRI defined regions (p=0.007). This increase in microglial activation from the levels observed in the normal appearing white matter likely contributed to the abnormal signal observed on MRI in this region of interest.

Furthermore, astrogliosis, as defined by an increased reactivity of astrocytes based on a GFAP stain, was observed in 12 of the 33 regions of interest (36%). Astrogliosis was often severe to the point of not being able to distinguish between different cells within the region of interest, unlike in normal appearing white matter, where each cell was detectable and resided in its own domain without overlapping between cells. The reactivity of astrocytes may have also
influenced the water movement within the MRI T2-only lesion area by restricting the movement and decreasing fractional anisotropy and therefore impacted the abnormal MRI signal. Finally, the blood-brain barrier integrity was evaluated in these T2-only regions of interest by staining for 3 serum proteins of different sizes, fibrinogen (340kDa), albumin (66.5 kDa) and IgG (~150 kDa), to determine if disruptions had occurred. The accumulation of serum proteins in the brain parenchyma or within cells of the brain would suggest that a disruption of the blood-brain barrier had occurred, resulting in edema (Fig. 10F). Serum proteins were observed in 30 of the T2-only regions suggesting that breakdown in the blood-brain barrier had likely occurred, which suggests that this in combination with the gliosis previously mentioned resulted in the MRI abnormal signal of the white matter in the absence of demyelination. These findings in the myelocortical MS patients T2-only MRI regions were very similar to what was observed in the classical MS patients T2-only MRI regions, except that more of the classical MS regions were demyelinated (22/40, 55%) and contained astrogliosis (37/40, 93%) (47).

7.1.2.3 MRI-defined T2T1MTR regions in myelocortical MS

The MRI regions that are abnormal on all MRI modalities (T2T1MTR) are often considered to be lesions with the most severe pathology. In the classical MS cases examined by Fisher and colleagues (47), 83% of the T2T1MTR regions contained demyelination. The 10 myelocortical MS patients had 31 regions that were defined by MRI as falling into the T2T1MTR category and 4 of these regions contained a small percentage of the region being demyelinated only on average 2.22% (Table 3); the other 27 regions were not demyelinated (Fig.
10G), which was expected given the lack of demyelination observed in these cases macroscopically.

Microglial activation was always present in all of the T2T1MTR regions and there was significantly increased density of class II staining per area (13%) compared to the density in the normal appearing white matter regions (p=0.003, Fig. 10H). The microglia had enlarged cell bodies and short thick processes, in a few of the regions the cells were rounded macrophages, but the exact function of these cells was not clear. The microglia could have been phagocytizing myelin or other debris cause by the disruptions to the blood-brain barrier. No specific pattern of microglial activation was observed in the T2T1MTR regions of interest between the 10 cases.

The T2T1MTR regions were also stained for GFAP to access the presence and extent of astrogliosis and whether these astrocyte changes were related to the MRI changes. Nearly half of the 31 T2T1MTR regions (15, 48%) contained astrogliosis within the MRI region of interest. There was more astrogliosis present in the T2T1MTR regions than the normal appearing white matter regions and T2-only regions, which is consistent with what others have shown, mainly that T2T1MTR lesions usually have a more severe pathological phenotype than the T2-only lesions. Overall, gliosis seems to be a strong correlate for MRI signal changes, since microglial and astrocyte activation are present in high percentages of T2-only and T2T1MTR MRI-defined regions.

Serum proteins were present in all of the T2T1MTR regions (Fig. 10I), suggesting that breakdown of the blood-brain barrier had occurred in the MRI abnormal region and contributed to the change in signal. The serum protein staining was often intracellular and based on the cell morphology was identified to be astrocytes. These cells are known for removing molecules such
as neurotransmitters from the extracellular matrix in gray matter, providing evidence that these cells remove molecules from the extracellular space and therefore could have also removed the serum proteins that had come into the brain parenchyma through disruptions to the blood-brain barrier. It is unknown if the serum proteins were removed from the extracellular matrix or if they were transported into the astrocyte cell bodies by the astrocyte end feet which comprise the parenchymal side of the blood-brain barrier. It is also unknown if the reactivity of the astrocytes was due to the disruption of the blood-brain barrier or if they became activated before the edema entered the parenchyma. In comparison with the 17% (7/41) T2T1MTR regions from classical MS patients, more of these regions were normally myelinated in the myelocortical MS patients (87%, 27/31), but both MS groups had increased microglial activation, higher incidences of astrogliosis, and evidence to support disruptions to the blood-brain barrier (47).
Figure 10. All images are from myelocortical MS MRI regions of interest. The normal appearing white matter by MRI contained normal myelin (A), little to no microglial activation (B), and the brain parenchyma was free of serum proteins like IgG (C). MRI lesions visible only on T2-weighted scans are free of demyelination (D), but did demonstrate increased microglial activation (E) and serum proteins in an intracellular pattern (F). Meanwhile, the MRI lesions evident on T2, T1, and MTR scans contained normal appearing myelin (G), extensive microglial activation (H), and serum proteins in intracellular and diffuse staining patterns (I).

Since there were significant differences of axonal counts and area were significantly decreased in the T2T1MTR regions, as compared to the MRI-defined normal appearing white matter regions from the classical MS patients examined by Fisher and colleagues (47).
addition, the axonal diameter index was greater in the T2T1MTR regions compared with the normal appearing white matter for the classical MS patients. These observations lead to the examination of axonal densities, counts and diameter measurements of the normal appearing white matter (Fig. 11A) and T2T1MTR defined regions (Fig. 11B) in the myelocortical MS patients. The normal appearing white matter from the myelocortical MS patients had similar mean axonal area and axonal counts as the T2T1MTR regions (Fig. 11C), which was surprising given the differences observed by Fisher and colleagues in the classical MS patients (47). However, there was a significant difference when the axonal diameter was compared between the normal appearing white matter regions and the T2T1MTR regions of the myelocortical patients, with the T2T1MTR regions having larger axonal diameters suggesting that swelling of axons is occurring even though there was not a significant loss of axonal number or demyelination in the abnormal MRI regions (Fig. 11C). Furthermore, when the myelin densities were compared between these two MRI defined region types there was no difference observed, further demonstrating the lack of myelin loss in the cerebral white matter of these myelocortical MS patients, even in the abnormal MRI regions (Fig. 11C).
Figure 11. Axonal staining with SMI 31 and SMI 32 of MRI defined normal appearing white matter (A) show a similar number of axons but slightly smaller axonal diameter compared to the MRI defined T2T1MTR regions (B). The densities of positive staining for myelin and axons were similar between the MRI normal appearing white matter regions and the T2T1MTR regions; however, there was a significant difference between the two region types for axonal diameter (C).

The last group of MRI regions of interest was the heterogeneous regions of which there were 13 MRI regions in this category from 7 of the 10 myelocortical MS brains in this study. These regions of interest were quite unique because the entire MRI abnormal signal was not either T2-Only or T2T1MTR, but rather had one portion of the region of interest which was T2-Only and another portion which was T2T1MTR. Majority of these regions had larger T2T1MTR portions than T2-Only (9/13) and all of the T2-Only portions were present at the edges of the region of interest. Ten of the 13 regions (77%) were myelinated, and like the other abnormal regions the 3 which did have demyelination only affected a small percentage of the total region of interest (16%). The general lack of demyelination in these regions was accompanied by microglial activation in all of the 13 regions and the density of class II staining per area (15%) compared to the density in the normal appearing white matter regions was not statistically
significant, but given a higher number of these regions of interest would likely have been significant (p=0.058). Astrogliosis was present in 9 of the regions (69%), with two-thirds of those regions containing astrogliosis being normally myelinated. All of the 13 regions contained evidence of serum proteins being present and were a mix between diffuse and intracellular staining patterns. This type of MRI region was not included in the analysis of classical MS cases in the Fisher and colleagues paper (47), therefore no comparisons can be made with these findings from the myelocortical MS cases.

Collectively, the combination of gliosis (from microglia and astrocytes) and disruptions to the blood-brain barrier in concert with axonal swelling contributed to the abnormal changes in MRI signal for the myelocortical MS patients. Demyelination was rarely present in these MRI defined white matter lesions, which confirms the macroscopic findings and suggests that although MRI is sensitive to MS-related cerebral white matter changes, it is not specific for any particular MS related pathology, and that these MRI lesions can arise without demyelination. These data raise an important point that not all MS MRI changes can be assumed to be demyelinated, even those which are more chronic T2T1MTR lesions.
Magnetic Resonance Imaging Guided Histopathological Analysis of Myelocortical Multiple Sclerosis White Matter.

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<th></th>
<th>NAWM (N=46)</th>
<th>T2-Only (N=33)</th>
<th>T2T1MTR (N=31)</th>
<th>Heterogeneous (N=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelinated</td>
<td>100% (46/46)</td>
<td>97% (32/33)</td>
<td>87% (27/31)</td>
<td>77% (10/13)</td>
</tr>
<tr>
<td>Percentage of Region of Interest Demyelinated</td>
<td>0%</td>
<td>0.48%</td>
<td>2.22%</td>
<td>16%</td>
</tr>
<tr>
<td>Activated Microglia</td>
<td>80% (37/46)</td>
<td>93% (31/33)</td>
<td>100% (31/31)</td>
<td>100% (13/13)</td>
</tr>
<tr>
<td>Density</td>
<td>7.4%</td>
<td>10.1%</td>
<td>13.5%</td>
<td>14.6%</td>
</tr>
<tr>
<td>Astrocytosis</td>
<td>15% (7/46)</td>
<td>36% (12/33)</td>
<td>48% (15/31)</td>
<td>69% (9/13)</td>
</tr>
<tr>
<td>Serum Proteins</td>
<td>35% (16/46)</td>
<td>90% (30/33)</td>
<td>100% (31/31)</td>
<td>100% (13/13)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>2% (1/46)</td>
<td>15% (5/33)</td>
<td>13% (4/31)</td>
<td>15% (2/13)</td>
</tr>
<tr>
<td>Cellular</td>
<td>33% (15/46)</td>
<td>48% (16/33)</td>
<td>71% (22/31)</td>
<td>46% (6/13)</td>
</tr>
<tr>
<td>Cellular &amp; Diffuse</td>
<td>0% (0/46)</td>
<td>27% (9/33)</td>
<td>16% (5/31)</td>
<td>39% (5/13)</td>
</tr>
<tr>
<td>Mean % Axonal Area (SD)</td>
<td>17% (4.9)</td>
<td>20% (6.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Axonal Count (SD)</td>
<td>1903 (731)</td>
<td>1575 (699)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axonal Diameter in mm</td>
<td>0.001 (0.0006)</td>
<td>0.002 (0.001)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Magnetic Resonance Imaging delineated the white matter into 4 categories, which each had their own unique pathology associated with them.

7.1.3 Atrophy and white matter lesion volumes by magnetic resonance imaging

Magnetic resonance imaging allows for an in vivo view of the brain and the pathology occurring within. The MRI images of the myelocortical MS patients exhibited a lot of the same features that are present in the classical MS patients’ MRI scans, such as white matter hyperintensities both surrounding the ventricles and in subcortical white matter, as well as enlargement of the ventricles (Fig. 12A-B). These MRI features are very indicative of an MS diagnosis but not of the specific underlying pathology, which is necessary to differentiate between these two MS subtypes.
Figure 12. A MRI T2–weighted image from a classical MS patient (A) illustrates some of the hallmark indicators of MS pathology by MRI, including enlargement of the lateral ventricles, periventricular and subcortical white matter hyperintensities, and a widening of the sulci between gyri. All of the above mentioned features are also present in the T2-weighted MRI from a representative myelocortical MS patient (B). No observable differences were detected between these two MS cohorts by MRI.

The number of and volume occupied by MRI defined white matter lesions is often an outcome measure for MS clinical drug trials to determine whether a drug is effective at slowing disease progression. The myelocortical MS patients were identified based on their postmortem pathology, more specifically by their lack of cerebral white matter lesions, but could there be a difference that would possibly identify these patients pre-mortem by MRI? With this question in mind, MRI white matter lesion volumes were measured and compared between the myelocortical and classical MS patients. The white matter lesions volumes were measured on T2-weighted scan images, T1-weighted scan images, and MTR images, but surprisingly there were no
differences between these two MS cohorts for white matter lesion volume on any of these MRI modalities (p = 0.183, p = 0.151, and p = 0.06 respectively, Table 4). Although there were drastic differences between these two MS cohorts with respect to area occupied by macroscopically visible cerebral white matter lesions of 12cm$^2$ in the classical MS patients compared to 0.2cm$^2$ in the myelocortical MS patients, however, no differences were observed between the two MS cohorts with respect to the white matter lesion volumes by MRI. Ultimately, these results suggest that the changes on MRI are not sufficiently specific to the underlying pathology, such that the presence of multiple white matter MRI changes does not equate to the same amount of macroscopic white matter demyelination. The MRI white matter lesions may have occupied the same brain volume in the myelocortical and classical MS patients, but would the decrease in demyelination of the myelocortical MS patients’ white matter result in a decrease in overall atrophy? Therefore, to answer this question, brain volume was calculated and compared between the classical and myelocortical MS patients, as well as to age-matched living healthy controls.

7.1.3.1 Comparing Whole Brain Atrophy between Controls, Classical and Myelocortical MS

Whole brain atrophy has been shown to be one of the best predictors of future disability in MS patients (197). The whole brain atrophy measured from postmortem MRI scan shows how much damage had occurred in total over the entire disease course. Although there were major differences in amounts of demyelination within the myelocortical and classical MS patients within the cerebrum, surprisingly there was not a difference in the amount of whole
brain atrophy as measure by brain parenchymal fraction (Table 4). Brain parenchymal fraction was significantly decreased for both of the MS cohorts compared to age-matched healthy living controls. This suggests that demyelination may not be the major pathology resulting in the overall damage that is occurring in MS patients, but rather a substantial neurodegeneration with varying amounts of demyelination. Despite the lack of difference in whole brain atrophy, there was still the possibility of there being differences in the various types of brain matter given particularly the differences in the amount of demyelination of the cerebral white matter.

Since there were differences in the cerebral white matter demyelination between the classical and myelocortical MS cases, we anticipated that white matter atrophy, as measured by white matter fraction, would be greater in the classical MS patients compared to that of the myelocortical MS patients. Again, despite the differences in macroscopic cerebral white matter demyelinated lesions, the white matter atrophy was similar between the two MS groups and both were significantly different from the controls (Table 4). This surprising result suggests that the demyelination might not be the most significant contributor to the loss of white matter volume, but rather an axonal degeneration leading to white matter atrophy.

Gray matter atrophy is measured by gray matter fraction which gives an indication of the total tissue volume loss within all the cerebral gray matter regions. There was a difference in the amount of cerebral gray matter demyelination between the two MS cohorts, but not as drastic as the differences observed in the cerebral white matter lesions. Therefore, we had expected no differences in the overall amount of gray matter atrophy and this is precisely what we observed (Table 4). There was no difference observed for gray matter atrophy between the myelocortical and classical MS patients, and may suggest that the mechanisms of gray matter pathology are similar between these two MS groups. However, only the classical MS gray matter fraction was
significantly decreased compared to the controls, and the myelocortical MS patients only showed a slight trend.

The final type of atrophy examined between the myelocortical and classical MS patients and controls was measuring differences in overall cortical thickness. This measurement specifically examines if there is any loss of the total cortical volume of these MS patients which may have occurred due to cortical demyelination, cortical degeneration, or tract degeneration from white matter or spinal cord demyelination. Similar to all of the other atrophy measurements, there was no difference in cortical thickness observed for the two MS cohorts, and both MS groups were significantly different from the controls (Table 4). Therefore, the slight increase of cortical lesions within the classical MS cases over the myelocortical MS cases does not reflect the overall cortical damage occurring within these two patient populations. Several studies have shown in postmortem tissue that the neuronal loss occurs in both the demyelinating cortex as well as normal appearing cortex (51, 60, 199, 284) and is the likely largest contributor to cortical atrophy.
### Magnetic Resonance Imaging Characteristics of Myelocortical Multiple Sclerosis and Classical Multiple Sclerosis Patients.

<table>
<thead>
<tr>
<th></th>
<th>Myelocortical MS Median (Ranges)</th>
<th>Classical MS Median (Ranges)</th>
<th>Healthy Controls Median (Ranges)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2 Lesion Volume (CM³)</td>
<td>34.1 (5.6-61.9)</td>
<td>55.9 (7.6-103.1)</td>
<td>NR</td>
</tr>
<tr>
<td>T1 Lesion Volume (CM³)</td>
<td>7.1 (1.2-15.4)</td>
<td>12.8 (2.2-40.8)</td>
<td>NR</td>
</tr>
<tr>
<td>MTR Lesion Volume (CM³)</td>
<td>6.2 (1.5-14.3)</td>
<td>18.2 (2.1-52.6)</td>
<td>NR</td>
</tr>
<tr>
<td>Brain Parenchyma Fraction</td>
<td>0.802 (0.766-0.840) p=0.04</td>
<td>0.763 (0.702-0.856) p=0.0004</td>
<td>0.847 (0.774-0.875)</td>
</tr>
<tr>
<td>White Matter Fraction</td>
<td>0.303 (0.271-0.363) p=0.03</td>
<td>0.307 (0.267-0.326) p=0.02</td>
<td>0.337 (0.308-0.352)</td>
</tr>
<tr>
<td>Gray Matter Fraction</td>
<td>0.492 (0.450-0.547)</td>
<td>0.455 (0.402-0.542) p=0.02</td>
<td>0.510 (0.456-0.555)</td>
</tr>
<tr>
<td>Cortical Thickness</td>
<td>2.9 (2.45-3.25) p&lt;0.0001</td>
<td>2.8 (2.69-3.29) p&lt;0.0001</td>
<td>3.4 (2.95-3.57)</td>
</tr>
</tbody>
</table>

Table 4. Magnetic resonance imaging metrics of white matter lesion volumes and several atrophy measurements were surprisingly not statistically different between the two MS cohorts. P-values are ANOVA pairwise comparisons from healthy control brain volume measures.

In summation, there were no qualitative or statistically significant quantitative differences between the myelocortical and classical MS patients by the MRI metrics of white matter lesion volumes, or by whole brain, white matter, gray matter or cortical atrophy measurements. This suggests that the mechanisms of degeneration may be identical within the classical and myelocortical MS patients, but that the differences between these two cohorts lie in mechanisms of cerebral white matter demyelination. This observation and other theories are discussed in greater detail in the following chapter, as are the strengths and weakness of the above project.
Chapter 5

Section 8.1 Discussion

8.1.1 Summary of Major Findings

We have characterized a pathologically novel subgroup of MS patients that displayed a significant reduction in the number and size of macroscopic cerebral white matter lesions than is typically observed for MS patients on 1 centimeter thick coronal brain slices at postmortem autopsy, which we have called myelocortical MS. This analysis was performed to investigate the relationship of cerebral white matter lesions and cortical demyelination. Quantification of this macroscopic finding was confirmed in 12 of 97 MS patients (12%), a similar incidence rate to the primary progressive MS population (223). At the time of autopsy, these myelocortical brains had a significantly smaller number and smaller areas occupied by cerebral demyelinating white matter lesions as compared to 12 age-, sex- and disability score-matched classical MS patients. Cortical and hippocampal lesions were of similar size between the myelocortical and classical MS patients, although the cortical lesions were more prevalent, roughly 3 to 1 in the classical MS patients compared to the myelocortical MS patients. Spinal cord lesions did not respect gray matter-white matter boundaries and affected many of the major tracts for both MS cohorts. The severe ambulatory disability of myelocortical MS patients (median EDSS of 8.0) was likely a result of the severe spinal cord demyelination. The number of spinal cord lesions observed from the 4 levels of the spinal cord was identical between the classical and myelocortical MS cohorts.
but the lesion sizes were slightly larger in the spinal cord cross-sections of the classical MS patients for both gray and white matter lesions. However, gray matter demyelination exceeded white matter demyelination in both myelocortical and classical MS spinal cord at a similar ratio of 2 to 1, suggesting similar mechanisms of demyelination were occurring within the spinal cords of both groups. Spinal cord dominant MS has been described previously (224, 225).

While the extent of subpial demyelination was not determined in those studies, it is likely that many of those cases represent myelocortical multiple sclerosis. Overall, the myelocortical MS patients had a gray matter dominant form of demyelination as compared to the classical MS patients which did not show the same level of preferential demyelination of gray or white matter in the cerebrum. These pathological signatures of the myelocortical MS patients are quite distinct from other forms of MS and alternative MS diagnoses, such as neuromyelitis optica, a degenerative disease of both spinal cord and optic nerve due to an antibody against aquaporin4 on astrocytes. The scarcity of cerebral white matter lesions in myelocortical MS patients is particularly interesting due to the MRI abnormalities on the scans of the myelocortical MS patients, which showed that the MRI white matter lesion volumes did not differ between the two MS cohorts as shown in Table 4. Furthermore, MRI-defined white matter lesions in the myelocortical MS patients were not a result of demyelination for regions abnormal on T2-weighted scan only or for the more severe lesions, abnormal on T2-, T1-weighted scans and altered MTRs. Therefore, our studies establish that combined changes in T2 and T1 intensities and reduced MTRs do not reliably detect demyelinated brain white matter, but in some cases reflect myelinated areas with axonal swelling, altered blood-brain barrier, microglia activation, and astrocytosis, such as in myelocortical MS cases. This evidence strongly supports the concept that axonal damage may be occurring prior to demyelination. Lastly and quite surprisingly, the
measures of whole brain, white matter, gray matter and cortical atrophy were equivalent between the classical and myelocortical MS patients and were significantly different from controls, with the exception of gray matter atrophy. These data suggest that damage such as axonal and neuronal loss, resulting in volume loss were also the same between the two MS cohorts. This has serious clinical implications, since this suggests that the demyelinating lesions only contribute a minimal amount of the accumulated atrophy and that a global neurodegenerative process could be the primary pathology in MS. This hypothesis would explain the lack of effects on disease progression by the current anti-inflammatory drug therapies. Currently, there is no conventional MRI signature to distinguish between living myelocortical and classical MS patients, based on the methods used in these studies. However, with the use of a stronger magnet in the 7T MRI scanner or possibly by a voxel-based morphology analysis of the white matter, abnormalities may lead to the identification of the myelocortical MS patients pre-mortem.

8.1.2 Conclusions

Myelocortical MS is an unique and previously undescribed variant of MS characterized by a lack of macroscopic cerebral white matter lesions in the presence of spinal cord and cortical demyelination. These patients have similar clinical histories and radiological evidence for the MS diagnosis as classical MS patients, but ultimately it is the pathological differences that separate these patients into a new MS variant from the other previously described subtypes. The importance of the characterization of this subset of patients is two-fold; 1) information on the pathogenesis of cortical and white matter demyelination can be collected from studying these
unique patients, and 2) the impact on clinical trials by excluding these patients from immunomodulatory therapy trials may yield higher drug effects in the classical MS population, for which the drugs will be most effective. Many of the myelocortical MS patients had been placed on immunomodulatory therapies, only to be switch from one brand to another, suggesting the ineffectiveness of these drug types on the myelocortical MS patients due to their lack of inflammatory mediated demyelination specifically in the cerebral white matter. Since spinal cord and cortical demyelination was still substantial within the myelocortical MS patients despite the administration of immunomodulatory drug therapies, gives evidence for 1) cortical demyelination, like spinal cord lesions arise independent of the cerebral white matter lesions, and 2) there is a different mechanism of demyelination in the spinal cord and cortical regions compared to the cerebral white matter lesions. The myelocortical MS patients’ pathological signatures emphasize the role that neurodegeneration plays in the overall MS pathology and therefore the necessity of targeting a neuroprotective agent as a therapeutic.

The significant subpial and hippocampal demyelination in cortical areas associated with memory and information processing and therefore is the likely cause of the cognitive decline reported in the individuals with myelocortical multiple sclerosis as well as in classical MS (49, 226, 227). The size and distribution of subpial lesions were very similar in myelocortical and classical cases, which suggests that the vulnerability of those cortical regions is the same and that the mechanism of pathology is also likely to be similar. A current hypothesis regarding the mechanism of subpial demyelination is that meningeal inflammation in deep sulci secretes immune mediators, which invade the surface of the brain, cause subpial demyelination and possibly contribute to subcortical demyelination by cytokine diffusion (62, 63, 66, 228, 229). In postmortem tissue of patients with an early onset of the disease and a rapidly progressing disease
course, meningeal inflammation was found to be associated with areas of subpial demyelination (62-64, 66). In a recent study, it was observed that these regions of meningeal inflammation could be detected on 3T MRI with gadolinium enhancement, however of the 299 MS patients only 74 (25%) contained a foci of leptomeningeal enhancement (230). Two of the cases which contained leptomeningeal enhancement came to autopsy and the area of enhancement did contain T cells, B cells, and macrophages, flanking a subpial cortical lesion. However, the authors did not report the number of subpial cortical lesions that were present without inflammation in the nearby meninges, which makes assessing these results difficult as to the real association between subpial demyelination and meningeal inflammation. In addition, it is still unknown whether the inflammation preceded the subpial demyelination or accumulated as a result of the demyelination, and possibly contributed to the expansion of the lesion, since subpial demyelination remains mostly undetectable by MRI. Although it is an attractive theory, and meningeal inflammation has been associated with subpial lesions in a subset of MS patients, the subpial lesions in myelocortical or classical MS cases did not contain increased numbers of parenchymal T cells or B cells nor were the lesions associated with increased meningeal inflammation or B cell-like follicles. Further evidence against this immunological mechanism of subpial demyelination is that subpial demyelination does not occur in immune-mediated animal models of multiple sclerosis (265,261) and has not been identified in other human brain diseases (275). However, meningitis due to bacterial or viral infection leads to lymphocytes, neutrophils and macrophages within the meninges, cytokine production and a pro-inflammatory environment. Subpial demyelination does not occur in this inflamed situation (275). Meningeal inflammation may contribute to subpial demyelination in a subset of MS cases; however, it does not appear to be an essential requirement for subpial demyelination to develop, since it is only
evident in roughly 25% of MS cases. Nevertheless microglial activation is an occasional finding at the border of subpial demyelination (231) and currently it is unknown whether these cells are activated to remove the myelin or if they are playing a protective role within these regions. Until subpial demyelination and meningeal inflammation are modeled in rodents, this will remain a controversial theory in the field. Furthermore, if the meningeal inflammation is causing the extensive subpial demyelination or if the subpial demyelination is a secondary event to white matter demyelination of the white matter, then it seems that immunomodulatory treatments should decrease the number and extent of cortical demyelination. This would require more advanced MRI techniques to determine if there are changes occurring in the cortex despite the treatment with anti-inflammatory therapies. Since cortical demyelination is currently not detectable on conventional 3T MRI scans and only a small percentage are evident on techniques such as double inversion recovery sequence (127, 232) or normalized cortical magnetization transfer ratios (142), this remains a controversial question.

Leukocortical lesions were also absent in the myelocortical MS cortex but were present in the classical MS cortex, suggesting that the origin of this lesion type is within the subcortical white matter and continues to develop into the deep layers of the cortex. This provides evidence of leukocortical lesions having similar mechanism of demyelination as other cerebral white matter lesions, and possibly explains the observation that the cortical portion of leukocortical lesions have higher numbers of infiltrating peripheral immune cells compared to other cortical lesion types (51, 73). The initiation of the lesion in the white matter portion results in a massive infiltration, which may spill into the adjacent cortical tissue. One major question is why some subcortical white matter lesions abutting the cortex extend into it (15.6%, 236) while others remain only in the adjacent white matter. It stands to reason that immunomodulatory drug
therapies will reduce the number of this type of lesion, similar to their effects on other cerebral white matter lesions, since the mechanism of demyelination is likely to be identical.

The hippocampus is a unique archaeocortical structure consisting of a mixture of gray matter and white matter regions that is especially sensitive to multiple insults, including repeated influx of inflammatory cells (77, 78). The hippocampus is central in learning and memory function, especially episodic and anterograde memory processing, which are the most frequently impaired cognitive modalities in MS (76). This is significant to the MS population because 40-60% of MS patients present with cognitive deficits over their disease course (76) and for some MS patients the cognitive deficits begin at the clinical onset of the disease (130-132).

Hippocampal demyelination affected the majority of the myelocortical and classical MS cases (21 of the 24 cases). The dynamics and mechanisms of hippocampal demyelination remains to be established. Hippocampi from myelocortical cases had similar amounts of gray matter demyelination but significantly less white matter demyelination than hippocampi from classical MS cases, providing addition support for gray matter dominant demyelination in brains from individuals with myelocortical multiple sclerosis. This pattern of demyelination in the myelocortical hippocampi is of particular interest because it could suggest that multiple mechanisms may be required for the demyelination of both the white and gray matter regions of the hippocampus. Demyelination of the hippocampus reduces many important neuronal proteins, including those required for synaptic plasticity, glutamate homeostasis, and learning and memory (79). Since the myelocortical MS patients had cognitive impairments with very little to no hippocampal white matter demyelination, this would suggest that the demyelination with-in the gray matter greatly affects the neuronal integrity of the hippocampus, leading to the cognitive dysfunction in these patients.
Spinal cord demyelination was prevalent in both the classical and myelocortical MS groups. The ratio of gray matter to white matter demyelination was similar between the two MS cohorts, which supports the concept that the demyelination of the spinal cord is likely occurring in a similar fashion between the classical and myelocortical MS cases. One possible explanation for the classical MS patients developing slightly larger spinal cord lesions could in part be a result of the increased cerebral white matter lesions in the tracts, which in turn decreases downstream axonal firing. It has been shown that reductions in axonal firing have effects on oligodendrocyte progenitor cells and oligodendrocytes, and ultimately myelination through glutamate transporters (233). The loss of saltatory conduction in these tracts could have downstream effects on remyelination in the spinal cord by a reduction in the release of glutamate and hence a decrease in glutamate signaling to the oligodendrocyte progenitor cells to remyelinate the spinal axons. It is possible that reduced remyelination of the spinal cord axons in the classical MS cases may have given rise to the larger areas of spinal cord demyelination in this MS population. Since the myelocortical MS patients are devoid of cerebral white matter lesions within these white matter tracts, the axonal firing is preserved and therefore triggers oligodendrocyte progenitor cells and developing oligodendrocytes through GLT1 or EAAC1 glutamate transporters to remyelinate bare axons within the spinal cord. The activation of the oligodendrocyte progenitor cells through this manner may have resulted in more remyelination and therefore the slightly smaller regions of demyelination within the myelocortical MS spinal cords. However, spinal cord pathology is known to develop independently from the pathology occurring in the cerebrum, but that does not preclude the influence of one on outcomes of the other. Since myelocortical MS patients had an average of 44% of their spinal cord demyelinated and likely a substantial amount of spinal cord atrophy had also taken place, this may suggest that
the mechanisms giving rise to spinal cord demyelination are the same as those occurring in the cerebral cortex despite the lack of cerebral white matter lesions.

Whole brain atrophy of MS patients is considered to be a good predictor and correlate of disease progression. This is likely due to the fact that whole brain atrophy includes the damage occurring in the gray matter, which is not visible by conventional MRI, and the damage in normal appearing white matter which is excluded when white matter lesion counts and volumes are measured. The most interesting and surprising result of these studies was that compared to myelocortical MS patients, classical MS patients had 69 times more brain white matter demyelination, 2 times more spinal cord demyelination and 2.5 times more cortical demyelination, but these two cohorts had similar amounts of whole brain and brain white matter atrophy. This discovery raises the possibilities that spinal cord and subpial demyelination or a degenerative process unrelated to demyelination drive atrophy. Whole brain atrophy in the early stages of the disease has been shown to be equally contributed to by gray and white matter atrophy, but in the later stages of MS gray matter atrophy rates increase, while white matter atrophy rates continue at a steady rate (160). This could explain some of the similarities between the myelocortical and classical MS atrophy measurements, since at the end stages of the disease; the gray matter atrophy plays a stronger role in the accumulation of whole brain atrophy. The possibility of white matter atrophy being driven by a degenerative process and not by an inflammatory mediated demyelination would suggest that the amelioration of the aberrant inflammatory responses will not be effective in slowing the progression of the disease. Clinical studies of the multiple immunomodulatory drugs show a reduction of the relapses but patients still continue to progress into the later stages of the disease, supporting this theory. The gray matter and cortical atrophy were also similar in myelocortical and classical MS brains, again
despite the slightly higher incidence of subpial lesions in the classical MS brains. It has yet to be determined whether subpial demyelination is responsible for cortical atrophy, although data of regional cortical atrophy suggest that regions most strongly affected by cortical atrophy are the anterior cingulate, superior frontal cortex, superior temporal gyrus and left insula (234, 235), which are regions that are also frequently demyelinated in MS patients (236). This would suggest that cortical atrophy may be a result of subpial demyelination, but more studies directly aimed at investigating this relationship are necessary.
Figure 13. Schematic of the potential pathological mechanisms involved in the different multiple sclerosis variants.

8.1.2.1 Importance of the studies in management of MS:

As mentioned previously, the identification of myelocortical multiple sclerosis has important implications for clinical management and treatment of MS patients. New MRI T2 hyperintensities are considered to be proof of heightened disease and inflammatory activity,
which would lead one to expect the myelocortical MS patients to have minimal new T2 lesion activity. However, given the similar white matter lesion volumes by MRI, active disease processes are occurring within the white matter despite the lack of demyelination. The clinical follow-up of MS patients typically involves neurological examination for assessment of EDSS score change and only conventional brain MRI scans, which do not visualize either subpial cortical lesions or spinal cord lesions. Myelocortical MS patients would be hard to identify by these measures since these patients do have progression of disability on the EDSS, conventional MRI white matter changes, but no imaging of cortical or spinal demyelination which may better separate myelocortical MS patients from the rest of the MS population. For a small number of MS patients, their presentation is initiated with progressive cognitive dysfunction while containing a paucity of white matter lesions by MRI, consequently the myelocortical MS variant may clarify this discordance of disability accumulation in the absence of an MRI correlate. Ultimately, the best imaging modality to separate the myelocortical MS patients from the classical MS patients would be one that detects cerebral white matter demyelination.

Pertaining to clinical trials for new MS therapies, myelocortical MS could be a significant population for potential exclusion from particular drug trials. One of the key standard outcome measurements for phase II trials of MS therapies is a reduction in the development of new lesions on T2 and post-gadolinium T1 images in RRMS patients. The inclusion of myelocortical MS patients in trials for anti-inflammatory therapies may skew the data in a manner which results in the drug appearing ineffective; therefore, this imaging-based outcome would not be sensitive to the potential benefits of anti-inflammatory therapies. Similarly, in clinical trials as well as in clinical practice, overall T2 lesion burden is frequently utilized to assess disease progression and active demyelination; however, the T2 lesions in myelocortical MS almost never
represent white matter demyelination. Additionally, roughly 31% of MRI defined white matter lesions identified in the classical MS patients were actually normally myelinated. These findings question the feasibility of T2 lesion burden as a primary outcome measurement in both myelocortical multiple sclerosis and classical multiple sclerosis. Nevertheless, continuous brain atrophy is the recommended standard outcome measure for progressive MS trials. The similarity between brain, white matter, and gray matter atrophy seen in myelocortical and classical multiple sclerosis patients gives further support that this outcome measure as the best read out of disease progression since it remains valid in myelocortical multiple sclerosis.

An interesting aspect of this thesis work is the relative similarities between cortical and spinal cord demyelination but differences in cerebral white matter demyelination between the myelocortical and classical MS patients. This leads to the conclusion that MS may be a disease in which multiple mechanisms are occurring simultaneously and result in different regional distribution of demyelination. This is not the first suggestion that multiple mechanisms may be affecting the pathological distribution of demyelinating lesions. Prins and colleagues described differences between white matter and gray matter lesions, suggesting that the mechanism of demyelination for these two regions may differ in part due to the microenvironment of these distinct locations (237). The authors concluded based on the differences in inflammatory infiltration, particularly of lymphocytes, that demyelination is either a mechanism that “does not require leukocytes or that the inflammatory pathogenesis of white matter lesion formation differs from that of gray matter lesions, which is largely irrespective of immune cell infiltration.” We propose the possibility of two mechanisms of demyelination, the first being a highly inflammatory mechanism affecting mostly the cerebral white matter with infiltration of peripheral immune cells and active to varying degrees in classical MS patients but absent in the
myelocortical MS patients. The second simultaneous mechanism is one that is less inflammatory-mediated and results in an ongoing process of spinal cord and cortical demyelination for both the classical and myelocortical MS patients. Inflammation is observed in spinal cord lesions, but not to the extent observed in the brain and appears to be more in SPMS as compared to PPMS (297). The exact mechanism for the spinal cord and cortical demyelination is unknown; however, one possibility is that an imbalance of metabolism in either the neurons or the oligodendrocytes leads to the demyelination of these regions. The high energy demands of producing myelin sheaths have long been known. If the oligodendrocyte metabolism is compromised, this could lead to the production of abnormal myelin, which in turn is recognized by surveying microglia and removed in an effort to maintain homeostasis and protect the surrounding CNS tissue (72). The energy imbalance of the oligodendrocyte could also result in cell death, suggesting an oligodendrogliopathy form of demyelination in the gray matter and spinal cord. By focusing on the oligodendrocytes, this research would be beneficial for the axons/neurons in the process, since neurons need the tropic support from these cells to keep from degenerating. Initially protecting the oligodendrocyte may result in faster remyelination, if less cells are lost. Furthermore, prolonged trophic support for the axon would result in less damage to the axon in the long term. It is well known that gray matter lesions are more prevalent in the later stages of MS (238), which could be explained by an oligodendrogliopathy, since a threshold of metabolic imbalance would need to be reached before cell death would occur, and over an extended period of time enough cell death would result in the formation of a lesion. In addition, we have shown that remyelination is also more prevalent in the gray matter, which would be able to compensate for this cell loss early in the disease course but as this compensatory mechanism fails, demyelination would accumulate, particularly in regions of higher energy demand, such as
the cingulate gyrus, insular cortex, hippocampus, etc. In the previously mentioned regions where there is a high degree of functional connectivity, there is a higher energy demand than in regions with less functional connectivity. The higher energy demand leaves these regions more vulnerable to energy deficiencies (298).

Another possibility underlying the energy imbalance could be a down regulation of transporter (e.g. monocarboxylate transporters) of substrates for the Krebs cycle (lactate, pyruvate), which would put increased pressure on mitochondria of the neurons and axons and result in increased production of reactive oxygen species, such as nitric oxide. The reduced activity of the Krebs cycle would lead to decreased substrates for the electron transport chain. It was previously demonstrated that inhibition of the electron transport chain with Antimycin A caused a decline in the production of N-acetyl aspartate (239). The limited availability of N-acetyl aspartate will result in a reduced amount of acetate which is required for myelin lipid synthesis. Mitochondrial defects of the respiratory chain have not only been identified in axons, but also in oligodendrocytes and astrocytes from acute white matter lesions (240). Nitration of mitochondrial respiratory chain proteins was observed in EAE (241), which is a common finding in many other neurodegenerative diseases (242).

Neuronal mitochondrial changes may play a role in cortical demyelination, but it is even more likely that they cause the neurodegenerative processes in MS patients. If the cell soma is unable to provide the axon with healthy mitochondria, then the strain on the axon, particularly a demyelinated axon, from the redistribution of the Na+ channels will likely lead to the degeneration of the axon. Mitochondrial changes have been demonstrated in models of demyelination (243, 244), MS tissue samples (239, 245-248) and myelinated white matter models of hypoxia and ischemia (as reviewed in (249)). Mitochondrial changes range from
morphological changes such as swelling to reduced mitochondrial oxidative phosphorylation chain activity to mitochondrial DNA changes. Mitochondrial swelling was shown to develop prior to axonal swelling and degeneration in the absence of demyelination in an EAE mouse model (243). The cause of the mitochondrial swelling was predicted by the authors to have been a result of the inflammatory environment producing reactive oxygen species, and reactive nitrogen species. Mitochondrial DNA damage has also been predicting to result from the production of reactive oxygen and nitrogen species which would in turn reduce respiratory chain activity (250). Normal appearing and lesional motor cortex from MS patients was observed to contain neurons devoid of complex IV, as well as a decreased gene expression of 26 nuclear-encoded subunits of the respiratory chain in myelinated motor cortex, which corresponded with reduced activity in complexes I and III (245). Together, all of these data indicate that mitochondrial dysfunction, axonal swelling, and degeneration possibly precede demyelination, indicating a potential role in neurodegeneration.

The varying degrees of the two demyelinating mechanisms of the spinal cord/cerebral cortex and white matter in combination with a neurodegenerative process that would all be occurring simultaneously may result in the entire spectrum of MS variants. The patients with Marburg’s and Balo’s MS, which as mentioned earlier are the most aggressive forms of MS, likely have a high degree of activity of the neurodegenerative process and inflammatory mechanisms, while the metabolic mechanism of demyelination is likely present to a lesser degree in these variants (Fig. 13). The high activity of the neurodegeneration likely leads to the rapid deterioration and early death of the Marburg’s and Balo’s MS patients. These MS patients often also show several white matter lesions by MRI, therefore suggesting a highly active inflammatory mechanism of demyelination. For these patients anti-inflammatory therapies in
combination with a neuroprotective reagent would be the best therapeutic strategy for ameliorating both the inflammatory demyelination and neurodegeneration. In a similar fashion, primary progressive MS likely has a high degree of neurodegenerative processes occurring, again resulting in a fairly rapid decline, with a significant metabolic-induced demyelination affecting the cortex and spinal cord of these patients, more so than the inflammatory mechanism. Primary progressive patients often show normal brain MRI scans, but contain multiple spinal cord lesions (151). Since cortical lesions are rarely detected by conventional MRI scans, it is likely that the primary progressive MS patients do not in fact have “normal” cortex, but just that the pathology there is not detected. Therefore, the primary progressive MS patients would have cortical demyelination along with the spinal cord demyelination. The minimal number of white matter lesions shown by MRI, along with the ineffectiveness of anti-inflammatory therapies in these patients, supports the concept that inflammatory mechanisms of demyelination are low in these patients. Therefore, the best therapeutic strategy would be a combination of a neuroprotective agent in addition to a treatment effective at reducing the spinal cord and cortical demyelination. In this concept, RRMS would begin with a very active inflammatory mechanism alongside a neurodegenerative process that steadily progresses, in addition to a probably small amount of the metabolic demyelination occurring simultaneously. Then when the patient switches to the SPMS stage, the neurodegeneration continues as before, but there is a switch from highly inflammatory to a more prominent metabolic demyelination. This theory is supported by reports that new T2 lesions are frequent in the RRMS stage but are rare in SPMS (87-89, 251); therefore, the inflammatory process is present early but regresses later in the disease. In addition, there are numerous reports that cortical demyelination is more prevalent in the progressive stage of MS (190, 198, 238) but can also be present early, in particular
leukocortical lesions (7). This suggests that the activity of this pathology is ongoing throughout the disease. Moreover, brain atrophy has been identified even in clinically isolated syndrome patients, which is evidence of the neurodegeneration also taking place at the onset. These MS patients would require therapies to target all three pathological mechanisms from the onset to be most effective at slowing the accumulation of disability. Myelocortical MS patients probably have very little, if any, of the inflammatory mechanism driving the pathological white matter demyelination, but rather a substantial amount of the neurodegenerative and metabolic demyelinating processes transpiring, resulting in the accumulation of spinal cord and cortical demyelination in combination with atrophy. Similar to the primary progressive patients, treatments which would diminish the metabolic and neurodegenerative mechanisms would be most beneficial to the myelocortical MS patient population. Finally, the benign MS patients would be expected to have a small amount of each of these pathological mechanisms occurring, which may result in pathological symptoms after many years due to the normal healthy mechanisms compensating for some of the central nervous system damage, until age related changes reduce the brains’ ability to compensate. In these cases, it would likely be most beneficial to treat them with a neuroprotective agent, since the other mechanisms only very minimally have an effect on disability accumulation in these patients.

If this theory of multiple pathological mechanisms in MS patients is accurate, then it stands to reason that as the immunological modifying therapies become more specific for the inflammatory processes taking place in the white matter of classical MS patients, this will further limit the benefit to exclusively the classical MS patients. It also puts forth the possibility that the specific immunological modifying therapies will drive more patients to have a more myelocortical-like pathological phenotype. However, disability will still build up, as has been
demonstrated by the myelocortical MS patients, this second mechanism of demyelination, independent of the inflammatory effects, can be just as devastating in contributing to patient neurological disability. Therefore, it is just as important to understand the underlying pathology and mechanisms of the cortical and spinal cord demyelination, as well as brain atrophy, in identifying therapeutics to counteract the mechanism of progressive neurological disability of MS patients.

8.1.2.2 Impact statement

These studies have provided important clues to increase our understanding of the pathogenesis of MS. Identification of MS brains with a paucity of brain white matter demyelination has profound implications such as:

1) The separation of brain white matter demyelination from subpial demyelination in individual brains supports the concept that subpial and cerebral white matter demyelination occur by different mechanisms. Subpial demyelination is not a result of inflammatory mediators, neither from B-cell follicles, nor from infiltration of inflammatory cells, hence the hypothesis of a non-inflammatory mechanism. The different demyelination mechanisms suggest that the current immunomodulatory therapies are not likely to be effective in reducing cortical lesions. Therefore, other therapies such as neuroprotective agents and targets of remyelination which would affect all regions of demyelination, may be the best possible drug targets.
2) The absence of leukocortical lesions in myelocortical MS brains supports previous conclusions that leukocortical lesions originate in cerebral white matter and differ in origin from subpial lesions.

3) Spinal cord demyelination may occur by mechanisms that are shared by subpial demyelination and may be distinct from those of cerebral white matter demyelination. This provides further evidence of the necessity of investigating other therapeutic targets besides inflammatory modulating therapies.

4) Atrophy accrualment is independent of demyelination, as demyelination levels did not result in more atrophy, providing further support for the strong neurodegenerative component of this disease.

8.1.3 Limitations of the Thesis

One of the limitations with this work is the small sample size of the two patient populations, which may have prevented statistical significance from being reached in some of the analyses, such as the white matter lesion volumes measured on MRI. Although it is not possible to increase the number of patients in the study, examining more areas of the cortex and levels of the spinal cord would help determine if these observations of brain atrophy, spinal cord and cortical demyelination would change with increasing the “N”. A limitation of the methods is that all the histological analyses of lesions were performed on only one 30µm section for each region; therefore, the lesion area measured in this particular section may not completely reflect the extent of demyelination. Lesions do not orient themselves in any particular fashion, which
means that had the block been cut at a different orientation the lesion size may have increased or decreased based on this technique. The results were based on the section we took; however, the lesion could have been more expansive either anterior or posterior to the section examined. A volume of the lesions would provide a much more precise indication of whether, for example, the spinal cord lesions in the classical MS patients are actually bigger and not just oriented in cross-section while if the myelocortical MS lesions were more oval in shape they therefore are oriented longitudinally. However, this would require staining a section from multiple points in the 1 cm thick block to identify the beginning and the end of the lesion.

Another limitation of this study is that all conclusions were made based on the end resulting pathology and not by a longitudinal study over the disease course, which may have biased the conclusions toward the end stage mechanisms, which may not be the same as those at the onset of the disease. Only through the identification of these myelocortical MS patients pre-mortem could this be examined.

There is also a limitation in that the postmortem MRI scans across the patient population are performed over several decades and during this time there are upgrades to the system or the scanner which may have affected these analyses by becoming more sensitive to abnormal tissue changes after the software changes or maintenance of the scanner itself. However, the MRI scans of the classical MS patients were taken over a broader time range (2 more years) than those of the myelocortical MS group; therefore, the groups should be affected by these changes relatively equally. Since the classical MS cohort is the reference group, the marginally broader range in this data set makes for easier interpretation of the myelocortical MS cohort.
Despite the above mentioned limitations, this study is the first to identify and characterize the pathological phenotype of myelocortical MS and compare the pathology to classical MS pathology. This work is of great importance to the MS research community due to its implications regarding the underlying mechanisms of MS pathology, which suggest that neurodegeneration may be a primary pathological event with a possibility of a secondary aberrant immunological response that varies greatly in extent within the MS population, leading to some of the differences in the MS variants.

8.1.4 Recommendations for Future Research

As mentioned previously, many of the current therapies are immunomodulatory which affect the relapse rate, but do not stop the disease progression. Therefore, treatments which promote remyelination, inhibit cortical demyelination, and/or enhance neuroprotective mechanisms are most likely to yield desirable therapeutic effects and warrant further investigation. Hence the following studies are focused on these potential therapeutic targets.

8.1.4.1 Removal of Inhibitors of Remyelination

Endogenous repair mechanisms such as remyelination make good potential therapeutic targets for MS. Remyelination is the mechanism by which a demyelinated axon becomes myelinated again by a newly formed oligodendrocyte. Remyelination occurs spontaneously in
RRMS patients but eventually fails in progressive MS (308). Several theories have been proposed as to why this remyelination failure occurs; one theory is that the oligodendrocyte progenitor cell (OPC) recruitment becomes disrupted or depleted (310). Another hypothesis is that the differentiation from OPC to oligodendrocyte is inhibited (309, 310), possibly by factors within the microenvironment (58, 311, 312), such as extracellular matrix molecules, or inflammatory cytokines. The OPC density in chronic lesions has been shown to be reduced compared with the densities observed in normal appearing white matter (302), suggesting that OPC recruitment and migration were possibly affected. However, OPCs were not completely devoid in chronic white matter lesions which would suggest that differentiation into myelin-forming cells may also have been inhibited. MS WM lesions may contain inhibitors of OPC differentiation such as the glycosaminoglycan hyaluronan, which is increased within the white matter portion of leukocortical lesions, but not in the gray matter portion where remyelination is more successful (58).

Similarly, chondroitin sulfate proteoglycans (CSPGs) have been implicated to negatively impact remyelination (313). CSPGs are extracellular matrix molecules, composed of a polypeptide core with one or more covalently linked polysaccharide glycosaminoglycan side chains. The CSPG family consists of versican, aggrecan, phosphacan, brevican, neurocan, and NG2 (252). These macromolecules are the major component of the CNS extracellular matrix; during development CSPGs influence neurite outgrowth, cell adhesion, migration, axonal guidance and structural plasticity (253-255). The function of CSPGs in CNS injury may be more inhibitory to repair and regenerative processes. The expression of CSPGs rapidly increases during CNS injury (such as traumatic brain injury or spinal cord injury) and in glial scars (256).
CSPGs have not only been shown to increase in glial scars, but also to correlate with breakdown of the blood-brain barrier. It is has been hypothesized that the formation of the glial scar and increased expression of CSPGs is due to a combination of factors such as thrombin, IL-1 and CNTF in response to tissue damage (256). The glial scar in combination with the increase of many extracellular matrix molecules (including CSPGs) many seal off the lesion area to contain the damaged tissue and prevent further invasion of inflammatory cells into the brain parenchyma. Although this may be at first neuroprotective, it also inhibits the repair of the lesional area in the long-term by forming a chemical and mechanical barrier, preventing the migration and differentiation of the OPCs within the lesion. The different CSPGs are produced by the glial cells within the glial scar; astrocytes produce brevican, neurocan and phosphacan while oligodendrocytes produce NG2 and versican (252). All CSPGs except phosphacan have been shown to increase during CNS injury. It was demonstrated in a spinal cord injury model that the enzymes xylosyltransferase-I and –II, which produce CSPGs, were under the control of the SRY-related HMG-box transcription factor Sox9 (306). The xylosyltransferases-I and –II (XT-I and XT-II) along with chondroitin 4-sulfotransferase (C4ST) add the chondroitin sulfate side chains to the core protein. All three of the enzymes are transcriptionally regulated by Sox9 in primary astrocyte cultures and they increase expression following spinal cord injury (252). Therefore, in spinal cord injury, the astrocytes are likely responsible for the increase in CSPG expression, which may also be the case in MS white matter lesions. McKillop and colleagues demonstrated that when Sox9 is conditionally knocked out in a spinal cord injury model, there is reduced expression of CSPGs and improved hindlimb locomotor recovery (252). This recovery may be a result of a loss of the barrier for axons to grow through the injury or it may be related to
the loss of perineuronal nets, which allows for structural plasticity to occur and therefore more connectivity and functional recovery.

In MS tissue, different patterns of these extracellular matrix molecules are expressed depending on the microenvironment. The epicenter of acute MS white matter lesions demonstrate a reduction in CSPG staining as compared to control white matter (257). Some foamy macrophages have been shown to contain granules of CSPGs within the lesion center, suggesting that along with myelin debris, CSPGs are being cleared from lesions (257, 258). However active lesions had an increase in CSPG staining at the lesion border (257). Some astrocytes within the chronic active lesions also stained positively for CSPGs suggesting an interaction between these extracellular molecules and oligodendrocytes (257). In contrast, the chronic inactive lesions showed a decrease in CSPGs, suggesting that remyelination may be possible at later time points within the lesion staging, since a reduction of these inhibitor molecules would allow for more effective remyelination. In the normal appearing white matter adjacent to the lesion, Sobel and Ahmed described aggregates of some CSPGs within the extracellular matrix (257). This increase in extracellular matrix in the normal appearing white matter and border of the lesion may inhibit oligodendrocyte precursor cells from migrating into the lesion core, differentiating into mature oligodendrocytes and remyelinating the bare axons. This would explain the observation that white matter lesion remyelination is restricted to the lesion border. In the spinal cord similar patterns were seen with increases of CSPGs around inflammatory lesions and aggregates in the normal appearing tissue. Sobel and Ahmed hypothesized that the function of this upregulation of CSPGs around MS lesions may relate to their function in development as forming barriers within the CNS (257). CSPGs have been implicated in the formation of white matter tracts during development by creating restrictive
barriers and tunnels to guide axonal growth (259). CSPGs may be forming barriers in MS to block the expansion of the lesion and/or to inhibit axonal growth into the lesion area, while at the same time blocking the migration and possibly differentiation of oligodendrocyte progenitor cells and neural stem cells from entering the lesion to promote remyelination and repair. CSPGs in culture have been shown to inhibit OPCs from migrating and differentiating into mature oligodendrocytes (260).

Another potential marker to investigate as an inhibitor of remyelination would be the leucine rich repeat and immunogloblin-like domain containing protein-1 (Lingo-1). This single transmembrane protein is found on neurons and binds to the receptor NgR1 along with the co-factor p75 (279). Primarily Lingo-1 expression is in the prefrontal cortex, cerebellar cortex and the thalamus (282). This complex of Lingo-1 and NgR1/p75 interacts with oligodendrocyte myelin glycoprotein, MAG or Nogo on the oligodendrocyte to regulate myelination and results in neuronal survival signaling. Lingo-1 inhibits oligodendrocyte differentiation and remyelination through a homotetramer complex. An antagonist of Lingo-1 has been shown to ameliorate these inhibitor effects in multiple mouse models of MS (280). Biogen, one of the major pharmaceutical companies, has a phase II clinical trial in MS patients with BIIB033, an IgG1 monoclonal Lingo-1 antibody that appears to block the formation of the homotetramers of Lingo-1 and therefore inhibits the intracellular signaling within the oligodendrocytes (281, 282). It seems that this mechanism would allow for more effective remyelination to occur in the white matter lesions of MS patients.

In order to study mechanisms of remyelination while it is ongoing, the utilization of a mouse model of MS is required. A popular mouse model to investigate both demyelination and subsequent remyelination is by the use of the toxin cuprizone in concert with rapamycin to
prevent spontaneous remyelination. Cuprizone (bis(cyclohexylideneamino)ethanediamide) is a copper chelator, which when ingested with rodent chow induces demyelination of the CNS in mice, possibly due to a copper deficiency (261). This animal model of MS results in white matter demyelination particularly the corpus callosum, in addition to extensive gray matter demyelination of the neocortex, piriform cortex, and the hippocampus. Furthermore, spontaneous remyelination arises upon withdrawal of the cuprizone from the diet. One advantage of utilizing the cuprizone model is that anatomical demyelination is easily reproducible and synchronous, making these variables constant, unlike in the experimental allergic encephalomyelitis (EAE) model. I would propose to do the following: to feed cuprizone chow to Sox9 knockout mice to investigate the role of Sox9 and its transcripts in remyelination in the cuprizone mouse model.

Sox9 expression levels increase mostly within and at the edge of MS white matter lesions as compared to MS normal appearing white matter and control white matter (Fig. 14A-C). In MS cortical subpial lesions, normal appearing cortex, and control cortex there was no obvious difference in the amount of Sox9 expression (Fig. 14D-F). Similar nuclear staining was observed in demyelinated white matter from cuprizone-fed mice (Fig. 15A-D).
Figure 14. Sox9 expression in white matter and cortex of MS and control tissue. A) MS white matter lesions contained increased amounts of Sox9 nuclear staining compared with B) MS normal appearing white matter and C) control white matter. D) MS cortical lesions contained similar amounts of Sox9 nuclear staining as E) MS normal appearing cortex and F) control cortex.
Sox9 Staining in Cuprizone Mouse Model

Figure 15. The Cuprizone mouse model shows similar Sox9 expression as the MS tissue, with little expression in the corpus callosum white matter from a wild-type mouse (A), but after demyelination for 6 weeks Sox9 expression increases (B) and stays elevated after 12 weeks of demyelination (C) but decreases during periods of remyelination (D).

In a microarray analysis of leukocortical and distinct white matter lesions, Sox9 expression increased at the border and within the white matter lesions or white matter portions of leukocortical lesions. Meanwhile, Sox9 mRNA levels decreased in the gray matter portion of leukocortical lesions (Fig. 16A). These data are interesting because we have previously shown that remyelination is increased within the gray matter portion of leukocortical lesions as compared to the white matter portion. The increase of CSPGs following the increase in their transcription factor Sox9 within the white matter portion of leukocortical lesions may inhibit remyelination mechanisms. If the gray matter portion has a decrease in Sox9 and therefore a
subsequent decrease of CSPG expression, this could result in more remyelination in this portion of the lesion.

Western blot analysis of proteins from the cortex of cuprizone mouse model of MS show a decrease of Sox9 expression during demyelination, which likely allows for the spontaneous remyelination and remains low during recovery (Fig. 16B). It would be expected that given a longer recovery period to the point of complete remyelination that Sox9 expression would return to baseline levels. This demonstrates a significant reduction in the levels of Sox9 from baseline levels after 12 weeks of the cuprizone diet (p=0.004) and during recovery (p=0.002) which would allow for the extensive remyelination that is observed in the cortex (Fig. 16C). However, there was no significant difference in the Sox9 levels between 6 week cuprizone-fed mice and wild-type mice, even though there was a slight decrease. Sox9 expression levels after 12 weeks of the cuprizone diet and during recovery were significantly decreased from the levels after 6 weeks of cuprizone (p=0.04 and p=0.05 respectively).
Sox9 Expression Pattern in MS White Matter and Gray Matter Lesions

Figure 16. A) Microarray data demonstrates that Sox9 message increases in MS white matter lesions but decreases in MS gray matter lesions compared to human normal appearing tissue. B) A western blot of Sox9 from proteins isolated from wild-type mice (CTL), mice on the cuprizone diet for 6 weeks (6WK), mice on the cuprizone diet for 12 weeks (12WK), and mice on the cuprizone diet for 6 weeks and then 6 weeks of normal chow diet (6WK Rec). C) Quantification of the western blot shows a reduction of Sox9 expression during demyelination and remyelination in a mouse model of MS. NAWM= normal appearing white matter, NLWM= near lesioned white matter, WML= white matter lesion, NLGM= near lesioned gray matter, GML= gray matter lesion, T1= leukocortical lesion, CA= chronic active.

Next MS tissue and tissue from cuprizone-treated non-transgenic mice were double labeled with antibodies for various cell types and Sox9 in order to identify the cells that were positive for Sox9. Markers for microglia, myelin and oligodendrocytes, lymphocytes and neurons did not co-label with the Sox9 nuclei (Fig. 17). Astrocytes however extensively co-labeled with the nuclear Sox9 cells in both the MS tissue and the cuprizone-treated mouse tissue (Fig. 18). Astrocytes have many functions to maintain homeostasis of central nervous system tissue, so it seems ideal that they are the cell type which would produce the enzymes responsible for the production of the extracellular matrix.
Elimination of Cell Types Not Expression Sox9

Figure 17. A) Neurons labeled with HuR (green) did not colabel with the nuclei containing Sox9 (red). B) Microglia (Iba-1, green) also did not co-localize with Sox9 nuclei (red). C) Lymphocytes (LCA, green) were not labeled with the Sox9 marker (red). D) Increased expression of Sox9 (green) was present in the lesion area and at the lesion border by PLP (red).
These preliminary data suggest that Sox9 is upregulated in astrocytes within and at the border of MS white matter lesions. In contrast, there is a decrease in Sox9 expression in cortical gray matter lesions. These differences in Sox9 expression within the gray and white matter lesions may be responsible for the differences seen in the capabilities of remyelination within these two lesion types. In the gray matter, remyelination is more successful and this may be due to the reduced levels of Sox9 and its transcripts, xylosyltransferase-I and –II and chondroitin 4-sulfotransferase. The lower levels of these enzymes would reduce the amount of CSPGs produced within the lesion to inhibit OPC migration and maturation within these gray matter lesions. In contrast, the white matter lesions show increased levels of Sox9 and by a similar
logic would have increased amounts of CSPGs, which would ultimately inhibit and/or block the OPCs from migrating into the lesion and differentiating into mature oligodendrocytes. Therefore, remyelination would fail in the white matter lesions due to the inhibitory pathway of Sox9. This theory seems plausible since it has already been shown that CSPGs are increased in white matter lesions (257) and in vivo following lysolecithin demyelination in the spinal cord (260). Furthermore, CSPGs in vitro have been shown to inhibit OPCs from differentiating into oligodendrocytes (260). More recently it was demonstrated that aggrecan, neurocan, and NG2 have an inhibitory effect on OPC and oligodendrocyte process outgrowth and myelination, but these effects were reversed when chondroitinase ABC (enzyme which digests the polysaccharide glycosaminoglycan side chains) was administered to the cells (262).

The mechanism by which CSPGs could inhibit remyelination is thought to be due to the polysaccharide glycosaminoglycan side chains (GAG), similar to the phenomenon of inhibition of axon regrowth following CNS injury (263). The GAGs can also inhibit oligodendrocyte process growth (264) as well as myelination (262). This inhibition of oligodendrocytes is mediated through the receptor protein tyrosine phosphates (PTP) sigma which signals through the Rho-associated kinase (ROCK) pathway (262). This signaling likely prevents mature oligodendrocytes from myelinating the surrounding bare axons, halting the oligodendrocytes in a premyelinating state. Therefore, by inhibiting the CSPG production by knocking out Sox9 from astrocytes, we would predict that the reduced levels of CSPGs would allow for a more efficient progress in remyelination of both gray and white matter. Examination of myelin density at multiple time points (1, 3, 6 weeks) following the removal of cuprizone from the diet would identify whether remyelination is more efficient in Sox9-knockout mice compared to wild-type controls.
Other factors in addition to CSPGs may also contribute to the failure of remyelination in MS lesions. For instance, the inflammatory environment contains many cells and molecules that may also be inhibitory. For example, proteolytic and lipolytic enzymes, reactive oxygen or nitrogen species, and excitotoxins could be damaging to oligodendrocytes and possible induce cell death (265). This would need to be taken into consideration as inflammation is highly present in MS white matter lesions.

It has been previously shown that conventional Sox9 knockout mice as homozygous (Sox9+/−) and heterozygous (Sox9+/-) are embryonic lethal (266). Therefore, in order to study the effects of Sox9 loss-of-function on remyelination in adult mice, a tamoxifent-inducible conditional Sox9 knockout mouse line will be used. A mouse strain containing floxed Sox9 (loxP sites around exons 2 and 3) alleles will be bred to a transgenic mouse line of Cre recombinase under the promoter of glial fibrillary acidic protein (GFAP) expressing the Cre-estrogen receptor (Cre-ERT2) fusion protein. This breeding will result in Sox9 deletion in GFAP expressing cells throughout the CNS of the offspring after tamoxifen injections.

Tamoxifen will be administered through intraperitoneal injection daily for 4 days at a dose of 0.001mg/kg to all Sox9 flox/flox;Cre mice and heterozygous Sox9 flox mice injected with vehicle at 6 weeks of age. Once the mice reach 8 weeks of age, they will be treated with a combination of 0.3% (w/w) cuprizone supplemented rodent chow \textit{ad libitum} and intraperitoneal injections of 10g/kg body weight/day rapamycin for 6 weeks or 12 weeks. Heterozygous Sox9 flox mice will receive rapamycin injections only. Two groups of mice will be taken off the cuprizone/rapamycin treatment after 6 weeks or 12 weeks and given normal rodent chow for 1, 3 or 6 weeks in order to study remyelination.
Immunohistochemistry will be performed as previously described in order to quantify the amount of demyelination and remyelination that has occurred following the removal of cuprizone treatment. Immunofluorescence will be used to confirm the knockout of Sox9 protein from astrocytes in the Sox9 flox/flox;Cre mice. Western blot and PCR analysis will be performed to confirm the knockout of Sox9 message and protein levels, along with a decrease in its transcripts in the Sox9 flox/flox;Cre mice. Immunohistochemical staining for proteolipid protein will be analyzed by a myelin density measurement and counts of premyelinating oligodendrocytes based on morphology to be compared across time points from the control and Sox9 knockout mice for differences in the remyelination rate. Behavioral testing will also be performed, such as the Morris water maze and Y maze, in order to provide data on cognition and memory following Sox9 knockout.

8.1.4.2 Mechanism of Cortical Demyelination

Cortical demyelination is a prominent pathology in MS brains; however, the exact mechanism of this particular pathology is unknown. In this thesis work, it was demonstrated that B-cell follicles were not necessary for the development of demyelination in the cerebral cortex; therefore a mechanism separate from peripheral immune cells is likely the mechanism. Microglia are the resident immune cells of the brain, and have been shown to phagocytize debris (72), their role in cortical demyelination is unknown. Therefore, to elucidate whether the mechanism of cortical demyelination is dependent on the phagocytic action of microglia, examination of demyelination by cuprizone in the absence of microglia should be performed.
We hypothesized that the ablation of microglia prior to cuprizone-induced cortical demyelination will result in reduced cortical demyelination. Testing this hypothesis will be accomplished by utilizing the colony stimulating factor -1 (CSF-1) inhibitor (267), which will block the CSF-1 receptor on microglia resulting in apoptotic death of the cells. The mice will then be place on a cuprizone diet for 6 weeks to induce cortical demyelination, the sacrificed to measure the remaining myelin densities for alterations in demyelination capacity in the absence of microglia. In these novel experiments, we expect to find reduced levels of cortical demyelination due to the loss of phagocytic action of activated microglia. This predicted result would allow us to conclude that microglia play a key role in the remove of myelin from cortical axons in this toxic mouse model of cortical demyelination, and furthermore that this is likely the mechanism of cortical demyelination in MS subpial lesions.

Another unanswered question is: Does subpial demyelination occur by a thinning of the myelin in the cortex or by a wave of demyelination, which commences at the pial surface and radiates down the layers of the cortex? We would hypothesize that subpial demyelination develops by a global thinning of the myelin similar to the cortical thinning that occurs in normal aging primate cortex, however at an accelerated rate. In order to answer this question, we will take two approaches, the first will be to examine the formation of cortical demyelination at multiple, early time points in cuprizone treated mice, and secondly, we compare the myelin density of cortical regions from MS hemispheric sections to age-matched healthy controls.

Cuprizone causes extensive demyelination in the rodent brain, affecting both gray and white matter. Our lab has previously administered cuprizone to rodents to investigate demyelination and remyelination. However, demyelination was only examined after 6 weeks of cuprizone diet, and hence the pattern of the demyelination in this animal model has yet to be
compared to the suspected formation of cortical subpial lesions. Using the cuprizone mouse model of demyelination, we will examine the development of subpial demyelination, by immunohistochemistry, at multiple time points (1 week, 3 weeks, and 6 weeks) to determine the course of action occurring in the cuprizone-fed mice as well as in postmortem MS brains. Myelin density measurements from different layers of the cortex will be analyzed for changes across the 3 time points to determine where and when the demyelination is occurring.

In human hemispheric tissue sections stained for PLP, myelin density measurements will be performed and compared between MS and age-matched controls. The hypothesis for these studies is that in normally healthy individuals, a decrease in myelin density occurs with age in the upper layers (I-IV) of cerebral cortex; however, in MS this process is accelerated possibly due to an oligodendrogliopathy, resulting in cortical demyelination. Oligodendrogliopathy has been proposed in MS pathogenesis before, but most recently as one of the types of white matter demyelination under the Lucchinetti criteria (268-271). This is a reasonable conjecture since we often observed a decreased myelin stain in MS cortex (Fig. 19B) compared to controls (Fig. 19A), but not to a level that would be considered a lesion (Fig. 19C). Myelin density is decreased in the upper layers of the cortex from controls who died in their 5th decade of life compared to non-neurologically diseased controls that died in their 6th decade of life (Fig. 19D). These data supports the concept that myelin loss of the upper layers in the cortex occurs with age in normal controls. The upper layers of the MS cortex showed a decrease in myelin density compared to the age matched non-neurologically diseased controls (Fig. 19E), suggesting that rather than a wave of demyelination from the pial surface of the brain, cortical lesions develop due to a dispersed loss throughout the cortex.
Cortical Myelin Density for Multiple Sclerosis and Control Patients

Figure 19. Myelin loss is control and MS insular cortex. Myelin staining in a control patient shows a high density of myelinated fibers in cortical layers I-IV of the insular cortex (A). The myelin present in two age-matched MS patients demonstrates a slight decrease (B) or extensive decrease (C) in myelin density in the upper layers of the insular cortex. Myelin density in layers 1-2 and 3-4 from control insular cortex decreases with age (D). MS patients in their 60s contained significantly less myelin in all layers of the insular cortex compared to age-matched controls (E). Scale Bar = 200um. * p<0.05, ** p<0.005, *** p<0.0005.

Remyelination can be extensive in the cortex, therefore to determine the proportion of myelin which is a result of a newly formed oligodendrocyte remyelinating the bare axons of the cortex we will quantify the ratio of mature (Fig. 20A) to remyelinating oligodendrocytes (Fig. 20B) by morphological differences based on PLP staining. The ratio of mature to remyelinating oligodendrocytes was drastically different between the control and MS cortices in our preliminary data (Fig. 20C). Double labeling for myelin (PLP) and caspr will allow for the
measurement of intermodal length (Fig. 20 D-F), since remyelination is associated with shorter myelin sheath lengths and to determine if the oligodendrocyte ratio correlates with myelin density and internodal length.

Lastly, to investigate the possibility that subpial demyelination occurs as a result of a dying-back oligodendrogliopathy, we will examine the degeneration of the myelin sheath. If an oligodendrogliopathy did occur than the myelin sheath would show degeneration at the distal most end of the sheath. We predicted that the myelin associated glycoprotein (MAG) in the periaxonal membrane will disappear before PLP or myelin basic protein (MBP) in compact myelin. Examination of the myelin sheath by double-labeling control and MS subcortical areas with PLP and MAG antibodies to determine which are present. The majority of myelin sheaths in control cortex contain both PLP-positive internodes and periaxonal MAG staining (data not shown). However, in MS subpial cortex with reduced myelin density, many myelinated fibers contained both PLP (Fig. 21A) and MAG (Fig. 21B), while others are PLP-positive sheaths (Fig. 21C) with patchy MAG distribution (Fig. 21D), whereas other PLP-positive sheaths (Fig. 21E) have no detectable MAG (Fig. 21F). Interestingly there were also myelin sheaths negative for PLP (Fig. 21G) but positive for MAG (Fig. 21H) these are remyelinating the axons within the lesion area. These data support the hypothesis of dying back oligodendrogliopathy occurs in MS cortex.
Oligodendrocyte Morphometry and Internodal Length

Figure 20. Immunoreactivity for proteolipid protein (PLP) in OLs cell bodies identifies two populations: mature OLs (A, arrowhead) and newly myelinating OLs (B, arrowhead). The ratio of new OLs to mature OLs is greater in the MS cortex compared to control cortex (C); most of the new OLs are present in the upper layers of the cortex. Internodal lengths as determined by caspr immunohistochemistry in control (D, arrows) and MS (E, arrows) are significantly longer in control cortex compared to MS cortex (F). Scale Bars = 20um
Myelin Protein Degeneration

Figure 21. Myelin protein detection at the border of a subpial lesion. Most myelin internodes contain PLP (A) and MAG (B) and they are present along the entire myelin sheath. Other myelin internodes contained PLP (C) and patchy MAG (D) staining, PLP (E) and no MAG (F) staining.

8.2 Closing Remarks

Multiple sclerosis (MS) is an incurable inflammatory-mediated demyelinating, neurodegenerative disease of the human central nervous system, affecting millions of individuals worldwide. This disease is the major cause of non-traumatic neurological disability in young adults with a complex etiology. The combination of environmental factors and genetic susceptibility lead to the risk of developing MS but it is unknown how these factors orchestrate the onset, specific symptoms and clinical course of the disease, leading to the large spectrum of MS variants. Several variants of the disease have been described with different clinical, radiological, and pathological phenotypes, but the hallmark of all the MS variants is the demyelinating white matter lesions particularly those observed in the cerebrum. However, work
from this thesis demonstrate that one MS variant (myelocortical MS) is essentially devoid of the hallmark white matter lesions, despite MRI abnormalities suggestive of white matter demyelination and profound levels of disability. Although MS is generally considered a disease of the white matter, the cerebral gray matter, spinal cord and brainstem can also become demyelinated and contribute to the neurological disability of MS patients. Myelocortical MS patients contained demyelination in their cerebral gray matter, spinal cord and brainstem, along with MRI evidence for substantial brain atrophy similar to the levels of MS patients with white matter lesions, which is described in Chapter 4 of this thesis. This new MS variant is particularly important because it demonstrates that 1) cerebral white matter demyelination likely forms by a mechanism that differs from the mechanism resulting in cortical and spinal cord demyelination, 2) MRI changes are not specific for demyelination, but could be a result of myelinated axonal swelling, microglial activation and edema, 3) neurodegenerative processes are similarly active in MS patients with or without cerebral white matter lesions. Clinically, this patient population is important when considering clinical trials for immunomodulatory therapies, which would likely be ineffective to treat myelocortical MS, as well as emphasizing the importance of targeting neuroprotective therapies.
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