

University of Milan-Bicocca
School of Medicine and School of Science

PHD PROGRAM IN TRASLATIONAL
AND MOLECULAR MEDICINE
DIMET

**Quantitative Magnetization Transfer imaging
for in vivo analysis of myelin content
in experimental model of demyelination**

Dr. Laura Turati

Matr. No. 062727

Coordinator: Prof. Andrea Biondi

Tutor: Dr. Renato Mantegazza

Co-Tutor: Dr. Fulvio Baggi

XXVII CYCLE
ACADEMIC YEAR 2013-2014

Alla mia famiglia

TABLE OF CONTENTS

CHAPTER 1

1.1.1 Multiple sclerosis.....	8
1.1.2 Myelin architecture.....	15
1.1.3 Histopathological subtypes of multiple sclerosis.....	18
1.2.1 Experimental models of multiple sclerosis.....	22
- Experimental Autoimmune/Allergic Encephalomyelitis.....	25
- Theiler's Murine Encephalomyelitis Virus Induced Demyelinating Disease.....	28
- Shiverer mice.....	31
1.3.1 Cuprizone animal model.....	32
- Relevance of cuprizone model for clinical research.....	36
1.3.2 Gender and strain aspects in cuprizone model.....	37
1.3.3 Mechanisms of cuprizone-induced demyelination.....	38
1.3.4 Cell subtypes reactions during cuprizone-induced de- and re-myelination.....	39
- Oligodendrocytes.....	41
- Microglia and macrophages.....	45
- Astrocytes.....	48
- Neurons.....	50
1.3.5 Crosstalk between glia.....	52
- Cytokines and pro-inflammatory molecules.....	52

- Growth factors.....	53
- Chemokines.....	54
1.4.1 Magnetic resonance imaging.....	56
- Conventional magnetic resonance imaging.....	56
- Non-conventional magnetic resonance imaging.....	57
1.4.2 Magnetization transfer imaging.....	60
- Macromolecular pool size ratio and bound proton fraction.....	64
- Magnetization Transfer Ratio.....	64
- Ramani's signal equation and fitting.....	65
- Factors altering measured magnetization transfer values.....	67
1.4.3 Quantitative magnetization transfer imaging in experimental models of demyelination.....	67
1.5.1 Scope of the thesis.....	70
1.6.1 References.....	71

CHAPTER 2

In vivo quantitative magnetization transfer imaging correlates with histology during de- and re-myelination in cuprizone-treated mice...	91
---	----

CHAPTER 3

3.1.1 Summary and conclusions.....	144
3.1.2 Future perspectives.....	147
3.2.1 References.....	150
 Publications.....	 154
 Acknowledgments.....	 155

CHAPTER 1

1.1.1 Multiple sclerosis

Multiple sclerosis (MS) was firstly described in 1868 by Jean Martin Charcot as “*La sclerose en plaques*” (Charcot, 1868). Starting from this initial observation, many researches tried to identify the aetiology of this disease, but the cause is still elusive. MS is generally considered an autoimmune, inflammatory, chronic disease of the central nervous system (CNS) characterized by perivascular infiltrates, demyelination, axonal loss and gliosis. Plaques form mainly in white matter and produce a wide variety of neurological symptoms in patients, such as paresthesias, motor weakness, visual disturbance and incoordination. MS is one of the main causes of disability in young people in the western world, twice common in women, with onset usually at the age of 20-50 (World Health Organization, 2008).

Although the clinical course of MS is highly variable, the National Multiple Sclerosis Society identified four disease subtypes, in 1996:

- Relapsing-remitting MS (RRMS): clearly defined neurologic dysfunction (relapse), with partial or complete recovery (remission), and lack of disease progression between relapses. More than 80% of initial MS diagnoses are part of this subtype (**Figure 1**; Lublin and Reingold, 1996).
- Primary-progressive MS (PPMS): disease progression from onset, without relapses but with occasional plateaus, and temporary minor improvements allowed. Approximately 10-15% of patients belong to this group (**Figure 1**; Lublin and Reingold, 1996).

- Secondary-progressive MS (SPMS): initial RRMS course followed by progression with or without occasional relapses, minor remission and plateaus. It was estimated that approximately 50% of RRMS patients convert to SPMS after 10 years (**Figure 1**; Lublin and Reingold, 1996).
- Progressive-relapsing MS (PRMS): progressive disease from onset, with clear acute relapses, with or without full recovery; periods between relapses characterized by continuing progression (**Figure 1**; Lublin and Reingold, 1996).

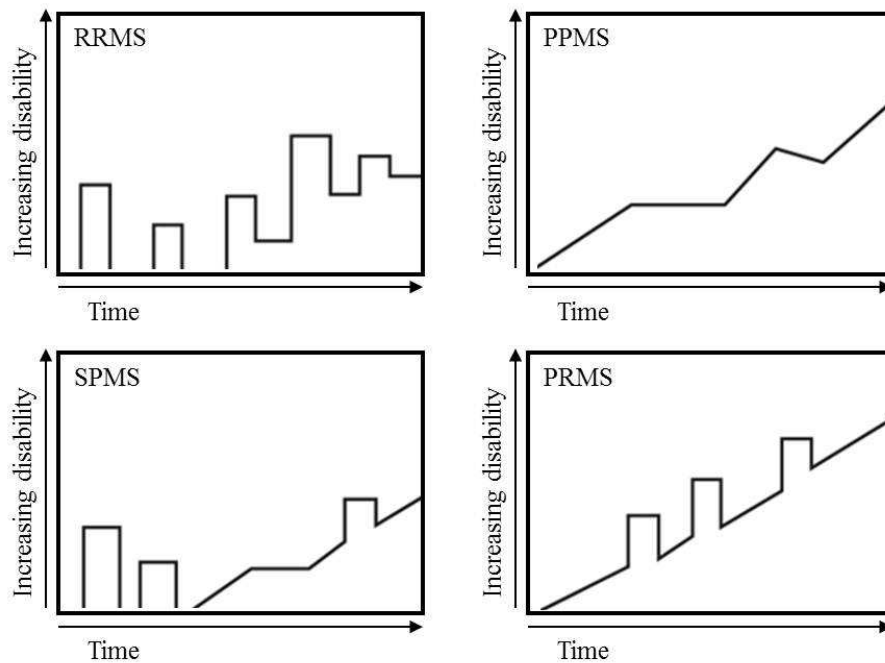


Figure 1. Clinical course of MS. Schematic presentation of the four MS subtypes based on the disease progression (Lublin and Reingold, 1996), defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology*. 1996 Apr;46(4):907-11).

Recently, thanks to magnetic resonance imaging (MRI) and analysis of fluid biomarkers, new MS phenotypes have been characterized. For example, the clinically isolated syndrome (CIS) was defined as the first clinical presentation of a disease that shows characteristics of inflammatory demyelination that could resemble a MS attack. In particular, CIS coupled with brain MRI plaques carries a high risk for MS development. Another emerging phenotype is the radiologically isolated syndrome (RIS), where incidental MRI findings suggest inflammatory demyelination in the absence of clinical manifestations. RIS may raise the suspicion of MS, that must be confirmed by clinical evidence of demyelination (Lublin et al., 2014; Milo and Miller, 2014).

Despite high heterogeneity in clinical manifestations, it is estimated that 2.1 million people suffer of MS worldwide (World Health Organization, 2008). MS frequency increases progressively with geographic latitude, being northern Europe characterized by the highest rates. Nevertheless some hot-spots of disease exist also in southern Europe, such as Sardinia in Italy. The pattern is similar in North America, with high presence of MS patients in Canada and northern United States (**Figure 2**; Kurtzke, 2005).

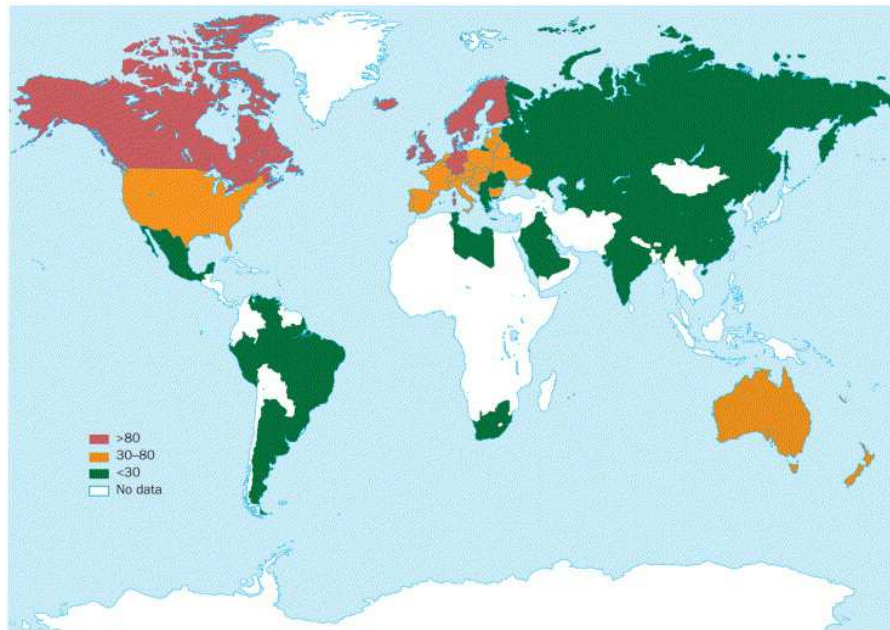


Figure 2. Worldwide prevalence of multiple sclerosis: incidence calculated in number of affected cases over 100000 persons (from Marrie RA. Environmental risk factors in multiple sclerosis aetiology. *Lancet Neurol.* 2004 Dec;3(12):709-18).

The incidence in different regions implies the influence of both genetic and environmental factors in MS development. The observation that there are extremely susceptible populations, such as Scotland (Kurtzke, 2005), immediately draws the attention on the genetic hypothesis. Another genetic evidence is carried by the observation that if a twin is a MS patient, him/her monozygotic twin has an increased risk of developing the disease (Hawkes and Macgregor, 2009). However the specific genes linked to inheritance remains elusive. Recent studies identified HLA-DRB1*1501 as MS susceptibility allele, causing altered interleukin (IL)-4 and IL-17 immune signalling pathways (Apperson et al., 2013). Nevertheless genetics alone cannot completely explain the aetiology of MS.

Indeed, environmental factors play a crucial role in MS destiny. Vitamin D is one of the most attractive hypothesis because can combine the latitudinal gradient related to MS frequency and its immuno-modulatory effects in experimental models. The major source for vitamin D is the exposure to sunlight but it is supplied also in the diet. Adzemovic and colleagues studied the effect of vitamin D supplementation in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune/allergic encephalomyelitis (EAE). Less severe inflammation, myelin loss and suppressed T lymphocytes responses towards MOG were observed only in juvenile/adolescent rats, suggesting that adolescence should be the target for vitamin D preventive treatment (Adzemovic et al., 2013). Another attractive hypothesis of MS aetiology is linked to Epstein-Barr virus (EBV) and other viral infections. In this context, two theories were originally proposed. The first theory, the poliomyelitis-hygiene hypothesis,

sustained that a viral infection contracted during early childhood should confer protective immunity, but late contact with the same virus should increase the risk for MS. The second theory, the prevalence hypothesis, postulated that the EBV infection is more common in the same regions with high MS frequency. EBV might explain many features of MS epidemiology, such as similar latitude gradient, but cannot justify the decline risk among migrants from high to low MS frequency areas (Ascherio and Munger, 2007; Kurtzke, 2005).

Despite epidemiological studies, the genetic and environmental factors at the basis of MS still remain elusive. Nevertheless, three main theories have been postulated in order to explain disease development (Milo and Kahana, 2010):

- MS is an autoimmune disease: it is more common in women than men (2:1 ratio); disease activity is suppressed during pregnancy and restart after childbirth; family members of MS patients show increased frequency of autoimmune diseases (Milo and Kahana, 2010).
- MS is an infectious-viral disease: perivascular infiltrates compatible with viral infections are often present in MS demyelinating lesions; other demyelinating diseases are known to be caused by virus (i.e. the subacute sclerosing panencephalitis, caused by measles virus infection); the time of exposure to the pathogen is crucial for the development of the disease (Milo and Kahana, 2010).
- MS is a neurodegenerative disease: axonal loss and neurodegeneration usually occur early in the course of the disease; in early MS plaques, microglia activation and oligodendrocyte apoptosis

appear before lymphocytes infiltration and demyelination (Milo and Kahana, 2010).

In conclusion MS is a multifactorial, inflammatory demyelinating disease, which results from interactions between genetic and environmental factors and further investigations will be needed to clarify its aetiology.

1.1.2 Myelin architecture

Myelin sheath is a multilamellar membrane wrapping neuronal axons in both the CNS and the peripheral nervous system (PNS), oligodendrocytes produce these plasma membrane extensions in the CNS, whereas Schwann cells fulfil the same task in the PNS (**Figure 3A and B**; Simons and Trotter, 2007, Min et al., 2009). Myelin is able to insulate the axons and to cluster the sodium channels in correspondence of Ranvier's nodes, allowing saltatory propagation of action potentials (Waxman, 2006).

Starting from morphological observations, myelin can be divided into compact and non-compact. The compact myelin is composed by an alternation of major dense layers of fused cytoplasmic membranes and light layers (intraperiod lines). The non-compact myelin is characterized by less dense structures and is localized at the edges of myelin cellular sheets (**Figure 3C**; Paz Soldán and Pirko, 2012).

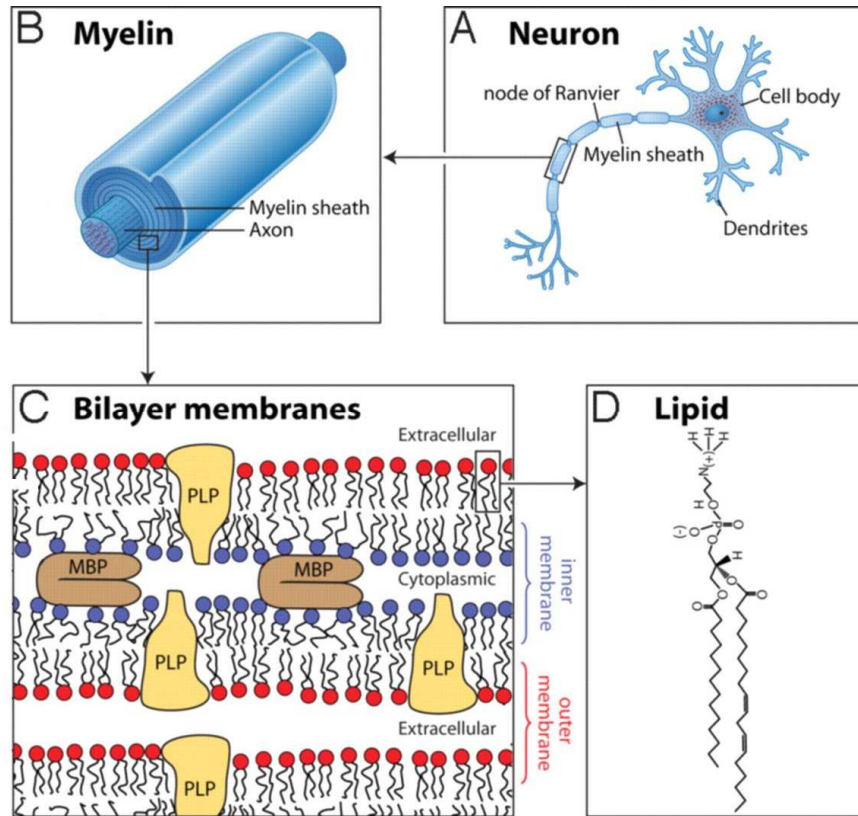


Figure 3. The structure of the myelin sheath. The myelinated axon (A), myelin sheath (B), bilayer membranes (C) and phosphatidylethanolamine (D) are shown (modified from Min Y, Kristiansen K, Boggs JM, Husted C, Zasadzinski JA, Israelachvili J. Interaction forces and adhesion of supported myelin lipid bilayers modulated by myelin basic protein. *Proc Natl Acad Sci U S A.* 2009 Mar 3;106(9):3154-9).

Dehydrated myelin has a peculiar composition characterized of ~75-80% lipids and ~20-25% proteins (Min et al., 2009). The glycosphingolipids (~31% of lipids), the cholesterol (~27% of lipids) and the phospholipids are the most represented lipids. The myelin bilayer has an asymmetric lipid composition, with specific localization of some lipids at the inner or outer membrane surface (**Figure 3C and D**; Paz Soldán and Pirko, 2012). Among the myelin proteins, proteolipid protein (PLP; ~50% of proteins) and myelin basic protein (MBP; ~30% of proteins) are the most abundant. Similarly to lipids, proteins display a specific distribution in myelin sheet. PLP is an integral membrane protein able to sustain the intraperiod lines, through electrostatic interactions between its extracellular domain and lipids (**Figure 3C**; Paz Soldán and Pirko, 2012). MBP is located between the two cytoplasmic faces acting as inter-membrane adhesion molecule and maintaining the major dense line (**Figure 3C**; Min et al., 2009). Many other proteins exist in myelin sheet, such as myelin associated glycoprotein (MAG), MOG and 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase).

Myelin is a complex structure able to physiologically overcome certain temporary tissue disturbances (i.e. transgenic mice lacking of a specific myelin protein are vital; Mikoshiba et al., 1990), but when normal myelin structure is terminally compromised, demyelination occurs (in terms of loss of myelin): this is the typical clinical manifestation of demyelinating diseases, such as MS.

1.1.3 Histopathological subtypes of multiple sclerosis

A complete histopathological characterization of heterogeneous MS lesions has been published by Lucchinetti and colleagues (Lucchinetti et al., 2000 and Lucchinetti et al., 2005). All MS patient lesions have in common inflammatory infiltrates composed of T-lymphocytes and macrophages, but they can be categorized into four different patterns (**Figure 4**).

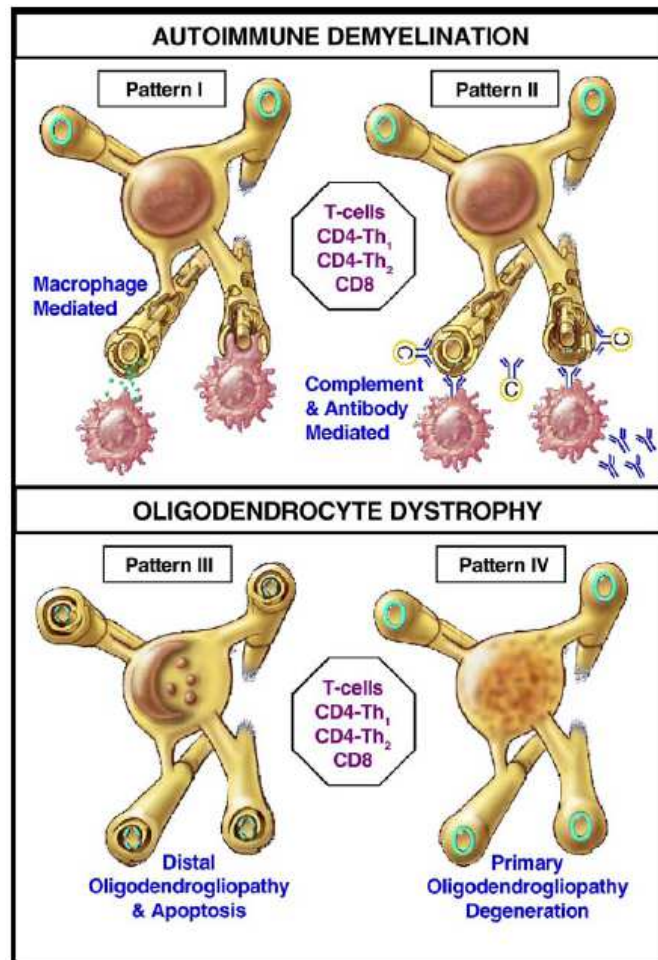


Figure 4. Immunopathologic patterns of demyelination. Schematic presentation of the four MS subtypes based on the underlying mechanism of myelin/oligodendrocyte destruction (from Lucchinetti CF, Parisi J, Bruck W. The pathology of multiple sclerosis. *Neurol Clin.* 2005 Feb;23(1):77-105, vi).

Pattern I lesions show active demyelination associated with T-lymphocytes- and macrophage-dominated inflammation (macrophage-associated demyelination; **Figure 4**). Pattern II lesions are characterized by deposition of immunoglobulin and complement activation at sites of active myelin damage (antibody/complement-associated demyelination; **Figure 4**). Plaques of pattern I and II are localized on small veins and venules and show sharply demarcated edges. Pattern III lesions display activated microglia, without deposition of immunoglobulin and complement activation. In this MS subtype, a rim of preserved myelin is observed around inflamed vessels. These lesions are characterized by a preferential loss of MAG protein and by oligodendrocytes undergoing apoptosis (morphologically visualized by condensed and fragmented nuclei). Consequently the active plaque border is characterized by a pronounced loss of oligodendrocytes and this pattern is also known as distal dying-black oligodendrogliopathy (**Figure 4 and 5**). Finally, pattern IV lesions contain T-lymphocytes, microglia and macrophages and reveal oligodendrocyte death (primary oligodendrocyte degeneration with secondary demyelination; **Figure 4**). Pattern II lesions are the most frequently observed in MS patients, followed in order of magnitude by pattern III, pattern I and pattern IV lesions (Lucchinetti et al., 2000 and Lucchinetti et al., 2005).

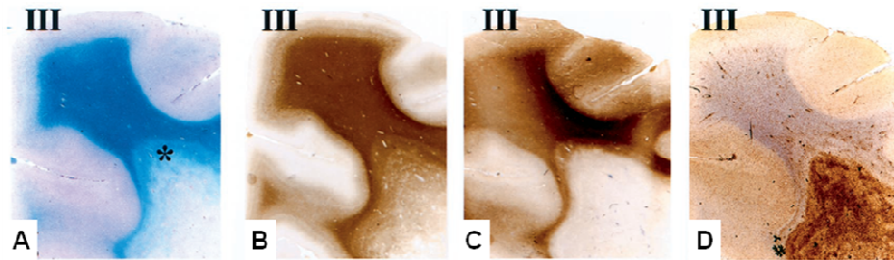


Figure 5. Acute MS (autopsy, male, aged 45 years, 3 weeks disease duration); pattern III; subcortical plaque with ill-defined borders and some concentric layering of demyelinated tissue. In comparison with Luxol Fast Blue (LFB) and MOG, there is a significantly greater loss of MAG in the lesions (asterisks). LFB (A); MOG (B); MAG (C); CD68 (D); original magnification, $\times 3$ (modified from Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol.* 2000 Jun;47(6):707-17).

1.2.1 Experimental models of multiple sclerosis

Experimental models are useful to clarify MS pathological processes and to study innovative therapeutic strategies. Four well-characterized experimental approaches to induce demyelination in animals exist: autoimmune inflammatory-induced demyelination (i.e. Experimental Autoimmune Encephalomyelitis, EAE), viral-induced demyelination (i.e. Theiler's murine encephalomyelitis virus induced demyelinating disease, TMEV-IDD), toxic demyelination (i.e. cuprizone animal model) and genetic myelin mutation (i.e. shiverer mice). Unfortunately, no experimental model by itself is able to represent the complexity of a heterogeneous disease, such as MS. Therefore, compatibly to the field of research interest, it is necessary to choose the animal model better mimicking the characteristics of disease that are selectively investigated, indeed each model shows typical advantages and disadvantages (**Table 1**; Gudi et al, 2014).

Induction	Animal models	Strength	Limitation
Auto-immune	EAE (induced by CNS tissue, myelin peptides, adoptive transfer of myelin reactive T cells)	- it mimics MS with respect to clinical symptoms and pathology; - it displays a relatively sensitive read-out system; - it can be induced in different animal species (mice, rats, rabbits, monkeys...); - it is mainly useful to test putative immunosuppressive and neuroprotective drugs, to study the behaviour of different T cell subtypes, mechanism of neuronal damage and loss, reaction of resident glial cells and their interplay with peripheral immune cells, to investigate the role of different molecular factors on T cell activation and to study BBB dysfunctions.	- it is an artificially induced sensitisation to myelin compounds; it typically requires adjuvants to activate the innate immune system; - high variety in susceptibility to EAE depending on species, genders and strains; - it does not reflect aspects of progressive MS; - the evaluation of remyelination is problematic since both de- and remyelination can proceed simultaneously; - the localization of lesions is unpredictable but mainly in the spinal cord.
Viral	TMEV-IDD (intracranial inoculum)	- useful for studying the phenomenon of epitope spreading; - useful to study mechanism of viral infection of neuronal and glial cells as well as viral persistence; - useful to study immune-mediated and virus-triggered demyelination but also to test different	- the assessment of remyelination is problematic since both de- and remyelination can proceed simultaneously; - TMEV is a slow-developing model and can be induced only in mice; - C57BL/6 mice clear TMEV; thus it is difficult to use

		regenerative, neuroprotective and immunosuppressive therapeutics.	genetically modified strains (transgenic colonies are mostly developed on the C57BL/6 background).
Toxic	Cuprizone (oral application)	- well reproducible, established model; - predictable kinetics of de- and remyelination; - simple induction protocol; - available as an acute and chronic demyelination model; - useful to study remyelination and cellular behaviour in the absence of peripheral immune cells.	- it is an artificial way of demyelination induction due to an irreversible damage of mature oligodendrocytes via a toxin of unknown mode of action; - does not involve T and B cell; - BBB remains intact; - no good clinical read-out; - not useful to study spinal cord demyelination, since demyelination occurs only in the brain.
Genetic	Shiverer mice	- consistency in myelination defects; - useful to study dysfunctions of myelination, neuronal and glial behaviour.	- no inflammatory model; - poor relevance for MS.

Table 1. Advantages and disadvantages of different MS experimental models (modified from Gudi V, Gingele S, Skripuletz T, Stangel M. Glial response during cuprizone-induced de- and remyelination in the CNS: lessons learned. Front Cell Neurosci. 2014 Mar 13;8:73).

- Experimental Autoimmune/Allergic Encephalomyelitis

EAE is the main model used to investigate the possible autoimmune origin of MS. In 1933, Rivers and colleagues described the phenomenon of experimentally-induced autoimmune encephalomyelitis for the first time, while elucidating the aetiology of neurological complications that followed anti-rabies vaccinations (Rivers et al., 1933). EAE can be induced using two different approaches: via immunisation using CNS homogenates, myelin preparations or purified myelin proteins/peptides (such as MBP, PLP, and MOG) in adjuvants; via adoptive transfer of encephalitogenic T cell blasts from EAE donors into naïve recipients (Schluesener and Wekerle, 1985; Sun and Wekerle, 1986; Kojima et al., 1997). The course of the demyelinating diseases include acute monophasic, chronic-progressive, relapsing-remitting; depending on species, strain, age and gender of animals; in addition to the type of induction (DeLuca et al., 2010). Pronounced weight loss precedes the clinical onset that is characterized by tail, hind- and/or forelimb paralysis and in more severe cases death. The development of the disease is fast and clinical signs are usually observable 2-3 weeks after induction (Schluesener and Wekerle, 1985).

Pathogenically, inflammation is more prominent than demyelination in EAE, in which small perivascular demyelinating lesions are mainly observed in spinal cord areas characterized by great ranges of immune infiltrates. Sub-cutaneous immunization leads to activation of autoreactive CD4⁺ T cells, specific for the immunization antigen, in the peripheral lymph nodes draining the area of injection site. These cells migrate out of the lymph node into blood circulation, and

manage to weaken the blood-brain-barrier (BBB) and extravasate into the CNS parenchyma, promote secondary recruitment of other immune cells (CD8⁺ T cells, B cells and mostly macrophages) and myelin destruction through the activation of resident (i.e. microglia) and infiltrating (i.e. macrophages) Antigen Presenting Cells (APCs). Moreover, infiltrating immune cells produce cytokines and chemokines and metalloproteases able to massively compromise the already leaky BBB and to recruit more immune cells in the site of inflammation. Within CNS parenchyma, encephalitogenic CD4⁺ T cells are de novo activated by self antigens that have been released by damaged myelin, phagocytosed, processed and presented to naïve CD4⁺ T cells, following the epitope spreading mechanism (**Figure 6**, Miller et al., 2007). Encephalitogenic T cells that mediate EAE are not exclusively CD4⁺. Indeed, Sun and colleagues demonstrated that adoptive transfer of MOG peptide-specific CD8⁺ T cells induce more severe and progressive form of EAE than active immunization of mice for EAE induction (Sun et al., 2001). In addition to inflammation and demyelination, neurodegeneration in terms of axonal damage can be observed in EAE animals. Acute and chronic axonal injury was found not only in the demyelinating lesions, but also in periplaque areas as well as in the normal appearing white matter. In contrast, no significant axonal damage was found in remyelinated shadow plaques (Kornek et al., 2000).

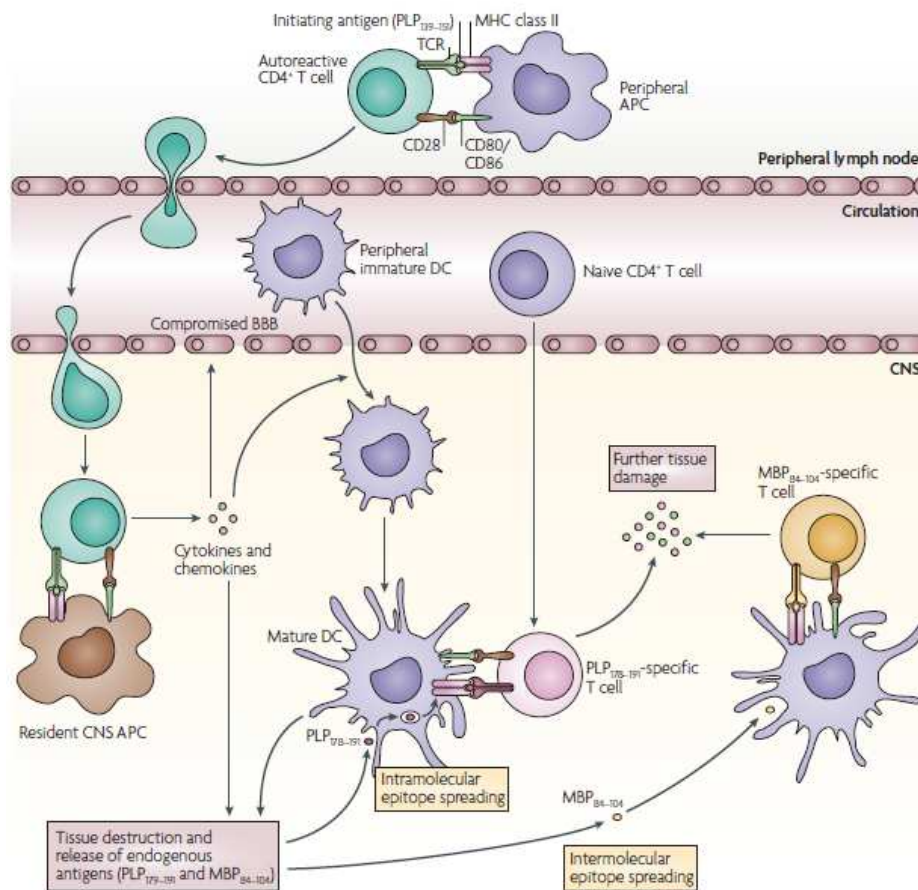


Figure 6. Pathogenic mechanisms involved in demyelination in the EAE model, the example of PLP peptide-induced relapsing-remitting EAE in SJL/J mice (Miller SD, Turley DM, Podojil JR. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. Nat Rev Immunol. 2007 Sep;7(9):665-77).

- Theiler's Murine Encephalomyelitis Virus Induced Demyelinating Disease

In 1937, Max Theiler isolated Theiler's murine encephalomyelitis virus (TMEV) from the CNS of mice with spontaneous flaccid paralysis of the hind legs (Theiler, 1937). TMEV is a positive single-strand RNA virus belonging to the genus *Cardiovirus* of the family *Picornaviridae*. Experimentally, TMEV-IDD is induced via intracerebral inoculation of the virus in susceptible mouse strains. The Theiler viruses can be divided into two subgroups: the first group is composed by highly virulent strains (i.e. GDVII and FA strains) that cause fatal encephalitis; the second group consists of less virulent strains (i.e. BeAn 8386 and DA), which induce mono- or biphasic disease and are used as viral murine model of MS. The monophasic disease consists of transient meningo-encephalomyelitis that reaches the peak within one week. In resistant mice (i.e. C57BL/6), CD8⁺ T cell-mediated clearance of the virus is completed in approximately 3 weeks. In susceptible mice (i.e. SJL/J), the infection persists and induces an inflammatory demyelinating disease causing lesions in the spinal cord, approximately 6-7 weeks after inoculum. Whilst demyelination progresses, complete hind limb paralysis occurs within 9 months from infection (Dal Canto et al., 1996). Susceptibility to TMEV-IDD is genetically linked to major histocompatibility complex (MHC) class I: H-2^{b,d,k} MHC haplotypes are resistant, H-2^{q,r,s,v,f,p} MHC haplotypes are susceptible (Rodriguez and David, 1985).

The exact pathogenic mechanism of demyelination in TMEV-IDD is not totally clarified. At the beginning of autoimmune response, APCs process and present antigens from TMEV or from self-tissue (i.e.

oligodendrocyte proteins or myelin), to T cells via the T-cell receptor (TCR) in the context of MHC class I or II molecules. Activated cytotoxic CD8⁺ T lymphocytes (via TCR/MHC I stimulation) directly damage the target. At the same time, activated T-helper (Th) cells (via TCR/MHC II stimulation) release proinflammatory cytokines that are able to recruit macrophages and microglia. Moreover, B cells secrete antibodies specific for foreign (virus) and self epitopes (**Figure 7**, Mecha et al., 2013). Two mechanisms of infection-induced autoimmunity are proposed for TMEV-IDD: molecular mimicry and epitope spreading. In the molecular mimicry model, cross-reactive T cells recognize both viral and self antigens, release cytokines and recruit activated monocytes and macrophages. In epitope spreading model, persistent TMEV infection activates specific T cells which mediate self-tissue damage, releasing self peptides which are then up taken by APCs and presented to self-reactive T cells (Vanderlugt and Miller, 2002). The TMEV-IDD model is characterized not only by demyelination, but also by axonal loss. According to inside-out lesion model, the lesions develop in the axon (inside) and extend to the myelin (outside), thus neurodegeneration represents the primary event, which is then followed by secondary demyelination (Tsunoda and Fujinami, 2002).

- Shiverer mice

Shiver mice are autosomal recessive mutants, characterized by a major deletion of the MBP gene, resulting in a severe reduction of myelin in the CNS. Electron microscopic observations reveal that these mutants have thin myelin sheaths, lacking of the major dense line, compared to wild type mice (Mikoshiba et al., 1990). Shiver mice show tremor during locomotion from 12 days postnatal, increasing in severity with age, associated with incoordination of the hind limbs and with death at approximately 4 months from birth.

This model cannot faithfully mimic the human disease but it is useful to study myelin repair. In particular, it has been used to evaluate the transplantation of myelinating-competent cells, such as neural stem cells and oligodendrocyte precursor cells (OPCs), and their ability to produce MBP and restore myelin (Duncan et al., 2011).

1.3.1 Cuprizone animal model

Cuprizone (bis-cyclohexanone-oxaldihydrazone) is a copper chelator discovered in the early 1950s, used for quantitative copper determination in biological samples. In the 1966, Carlton and colleagues demonstrated that this compound, when given systemically to animals, could induce growth retardation, interruption of pregnancy, cerebral oedema and hydrocephalus (Carlton, 1966). Cuprizone experimental MS model is appropriate to evaluate therapies able to block demyelination or promote remyelination. Indeed, young adult rodents fed with cuprizone develop demyelination in a reproducible manner, with well-known time and space windows. The reversion to normal chow diet allows remyelination. When cuprizone administration is continuous remyelination is abortive and demyelination persists (Matsushima and Morell, 2001).

This compound is able to induce demyelination in both rats and mice and the exact time course is dependent on its dose and age of treated animals. In particular, a dose of 0.1% (w/w) cuprizone is ineffective in the induction of extensive demyelination in 8-10 weeks old C57BL/6 mice, while 0.3-0.5% cuprizone induces severe weight loss and mortality. The higher doses (0.4 and 0.5, w/w) of chelator cause hepatotoxicity, characterized by an excessive numbers of multinucleated hepatocytes and the formation of megamitochondria in hepatocytes. Therefore, the 0.2% (w/w) cuprizone dose was suggested as the proper dose to induce demyelination without serious sides effects (Hiremath et al., 1998). A satisfactory explanation of age-dependent susceptibility to cuprizone is still lacking. However, experimental evidences demonstrated that juvenile (3 weeks) and

young-adult (6 weeks) mice show a greater demyelination, evidenced by reduction of MBP and loss of mature oligodendrocytes, and higher astrocyte activation compared to middle-age (8 months) mice after 6-week of cuprizone 0.2% (w/w) diet (Wang et al., 2013). On the other hand, axonal loss is more severe and remyelination is delayed or less efficient in older mice, probably due to the presence of aged OPCs (Irvine and Blakemore, 2006).

Starting from these observations, 0.2% (w/w) cuprizone is generally used to induce reproducible and extensive demyelination without side effects in 8-10 weeks old mice. In particular, C57BL/6 fed with cuprizone diet show different stages: early demyelination (1-3 weeks) and severe demyelination (3-5 weeks) that becomes complete following 5 weeks of treatment. If the cuprizone administration is reverted to normal chow diet at 5 weeks, early remyelination (5-6 weeks) is observed (**Figure 8**, Gudi et al., 2014). Cuprizone-mediated demyelination of encephalic white matter areas, such as Corpus Callosum (CC), has so far been the main target of most of the studies, but axonal sheath demyelination affect also gray matter, such as the cortex (Gudi et al., 2009) and hippocampus (Norkute et al., 2009). There are some differences between demyelination pattern of white and gray matter. In particular demyelination peaks 4.5 – 5 weeks of cuprizone feeding in CC, whereas it peaks at 6 weeks in the cortex and also myelin proteins degradation follows specific time-course (Gudi et al., 2009).

The cuprizone model is a toxin-induced CNS-intrinsic model of demyelination, characterized by intact BBB (Bakker and Ludwin, 1987) and no recruitment of peripheral T lymphocytes (Hiremath et

al., 2008). Cuprizone fed mice show weight loss, which reverts when mice return to normal chow diet. Moreover, cuprizone fed mice show diminished motor coordination and less exploratory activity than control mice (Franco-Pons et al., 2007; Ye et al., 2013).

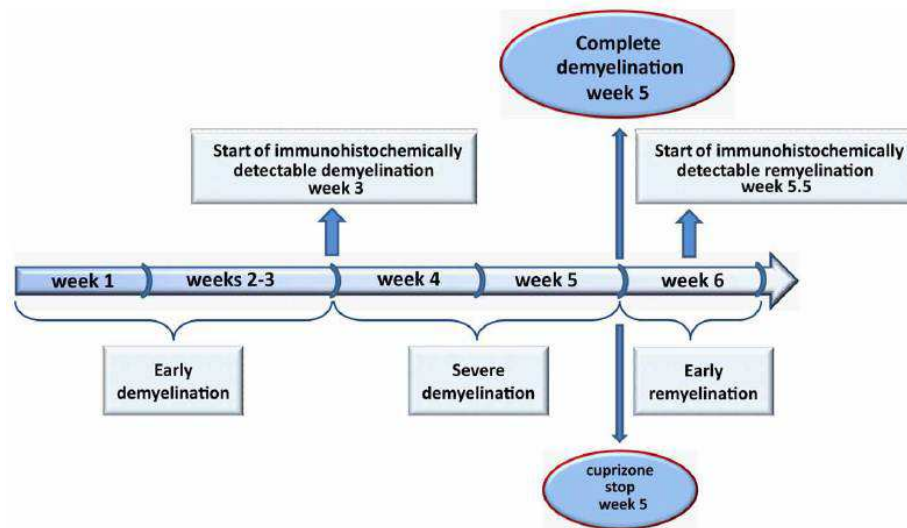


Figure 8. The course of de- and remyelination in the medial corpus callosum of 8-10 week old C57BL/6 mice fed with 0.2%(w/w) cuprizone (from Gudi V, Gingele S, Skripuletz T, Stangel M. Glial response during cuprizone-induced de- and remyelination in the CNS: lessons learned. Front Cell Neurosci. 2014 Mar 13;8:73).

- Relevance of cuprizone model for clinical research

Independent studies demonstrated that for MS patients with pattern III lesions oligodendrocyte apoptosis might be the first change in newly forming lesions (Lucchinetti et al., 2000; Barnett and Prineas, 2004 and Lassmann, 2007), maybe as a consequence of infection or damage mediated by some unknown toxin. In addition to extensive oligodendrocyte apoptosis, microglia activation and few or no T-lymphocyte infiltrations were observed in newly forming plaques (Barnett and Prineas, 2004). Many histopathological features of cuprizone-induced demyelination resemble pattern III lesions of MS patients (Torkildsen et al., 2008). However, other aspects of pattern III subtype, such as T-lymphocytes massive infiltrates, do not appear in cuprizone experimental model. This might be explained as a direct suppression of T-cell functions due to the toxin. Despite many studies, the mechanisms leading the oligodendrocyte death, in both cuprizone and MS lesions, are not completely clarified and further efforts should be made in this direction. However cuprizone experimental model is a suitable tool to study demyelination or remyelination events, leading to oligodendrocyte apoptosis.

1.3.2 Gender and strain aspects in cuprizone model

As pointed out above, clinical observation describes sexual dimorphisms in MS, with a higher prevalence in women (World Health Organization, 2008). Sex steroid hormones are elevated during pregnancy and play a neuroprotective action, reducing clinical MS manifestations (Dwosh et al., 2003). Acs and colleagues verify whether the administration of 17 β -estradiol and progesterone alone or in combination may influence myelin status during acute demyelination in cuprizone treated C57BL/6 male mice. 17 β -estradiol and progesterone alone administration partially ameliorated loss of oligodendrocytes and demyelination in the CC. The combined approach demonstrated a significant recovery of myelin, enhancement of OPC proliferation, astrogliosis and microglia activation, thus sustaining the efficacy of hormone administration in CNS myelination (Acs et al., 2009).

The role of gender/strain differences in cuprizone model has been better characterized by Taylor and colleagues, comparing C57BL/6 and SJL/J mice (Taylor et al., 2009; Taylor et al., 2010). The pattern of demyelination and remyelination is similar between male and female C57BL/6 mice and there is little or no difference in the loss of mature oligodendrocytes and subsequently OPCs proliferation (Taylor et al., 2010). Nevertheless, a major difference exists: SJL/J female mice are partially resistant to demyelination compared to male mice. Moreover, in this strain demyelination is slightly retarded and reaches its plateau after 7 weeks of administration, being completed at 10 weeks of exposure. Histopathologically, the demyelination of CC is also slightly different, indeed in SJL/J mice it is mainly located

immediately lateral to midline of CC, unlike C57BL/6 mice (Taylor et al., 2009).

The reasons of strain and gender variability in cuprizone-sensitivity are still not completely understood. Probably genetic background might be an explanation for the first and sex hormones for the second, but further studies are needed to clarify these aspects.

1.3.3 Mechanisms of cuprizone-induced demyelination

The mechanisms through which cuprizone- administration induce demyelination are not yet fully understood, but it is clear that its copper-chelating action modifies cellular processes. In particular, enlargement or clustering of normal mitochondria causes the formation of megamitochondria in the liver and brain of cuprizone-treated mice (Suzuki and Kikkawa, 1969). This is probably due to the alteration of some mitochondrial enzymes, which use copper as co-factor (such as monoamine oxidase and cytochrome c oxidase), happening during the first period of cuprizone exposure, prior to demyelination (Venturini, 1973). In presence of cuprizone and proinflammatory cytokines (IFN γ and TNF α), ex vivo glial cells show enhanced mortality and their mitochondria show marked activity decrease (Pasquini et al., 2007). However, the reason for the major vulnerability of mature oligodendrocytes to cuprizone in vivo is still unknown. An obvious explanation might be that these cells have to constantly produce and sustain myelin to wrap axons, and this effort requests mitochondria to function regularly. Indeed, oligodendrocytes are the predominant iron-reservoir in the brain that allows oxidative

metabolism and lipid synthesis for myelin production (Connor and Menzies, 1996).

1.3.4 Cell subtypes reactions during cuprizone-induced de- and remyelination

Different cell subtypes, including oligodendrocytes, microglia, astrocytes and neurons, orchestrate de- and remyelination events characterizing cuprizone model in a temporal/spatial defined manner (**Figure 9**).

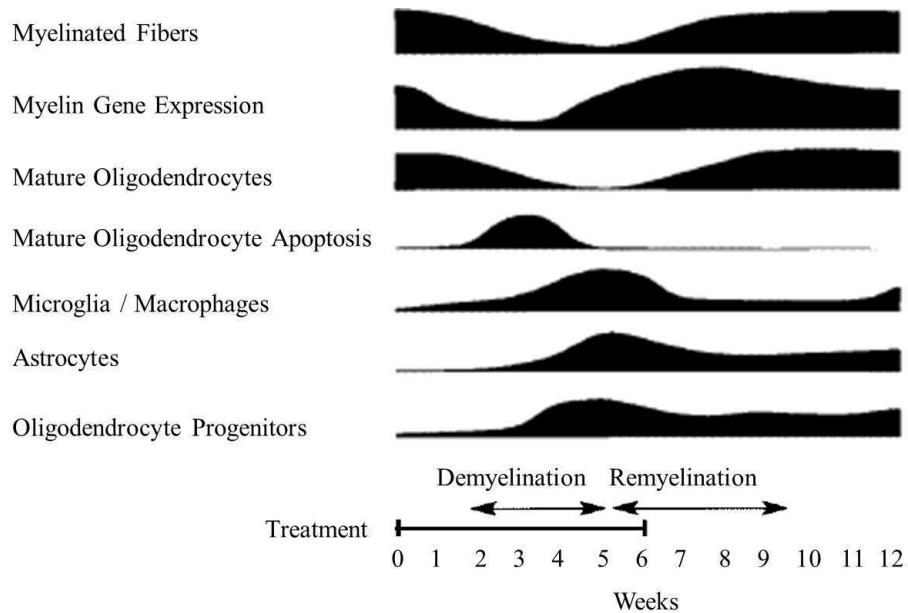


Figure 9. Summary of the cellular, morphologic and molecular events, during cuprizone-induced demyelination and remyelination in the CC of C57BL/6 mice (modified from Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol.* 2001 Jan;11(1):107-16).

- Oligodendrocytes

Oligodendrocytes produce myelin sheaths that isolate axons, allowing the saltatory conduction. Cuprizone exposure directly damages oligodendrocytes, which then undergo apoptosis within 1 week, therefore before demyelination events take place (**Figure 9**, Mason et al., 2000a). The expression of myelin specific genes is downregulated prior to acute demyelination. After 1 week of cuprizone diet, C57BL/6 mice mRNA values for MBP, MAG and Ceramide GalactosylTransferase (CGT) are already profoundly reduced with maximal depression at 3 weeks. mRNA levels return to normal values within 6 weeks, even if cuprizone administration is continued (**Figure 9**, Morell et al., 1998). In the early stage (6 and 10 days) oligodendrocyte loss is caspase 3-dependent, indeed dying myelin-associated neurite outgrowth inhibitor A (Nogo-A)-positive mature oligodendrocytes express activated caspase 3; whereas in later disease stages (21 days), a similar amount of oligodendroglial cell death is caspase 3-independent. During cuprizone treatment, also the gene expression level of first apoptosis signal (FAS), an activator of extrinsic apoptotic cell pathway, is up-regulated in the CC, following a time course similar to caspase 3. Transgenic mice (MOGicrefas^{fl/fl}) which lack FAS exclusively in oligodendrocytes, are nevertheless unable to prevent oligodendrocyte apoptosis (Hesse et al., 2010), thus oligodendrocyte death cannot be exclusively FAS dependent. Li and colleagues well characterized the transcription factor p53 as a pro-apoptotic molecule upregulated in the CC of C57BL/6 during the 3-4 weeks of cuprizone diet. Genetic (p53^{-/-} mice) and pharmacological inhibition (pifithrin- α treatment) of p53 allow less demyelination and

enhanced oligodendrocyte survival in cuprizone-fed mice (Li et al., 2008). After 3 weeks of cuprizone exposure, mature oligodendrocytes are depleted (Hesse et al., 2010) and demyelination can be detectable immunohistochemically (**Figure 10**).

Interestingly, in the CC remyelination starts very early, even before cuprizone suspension and before demyelination is detectable immunohistochemically. Neural glial antigen 2 (NG2)-positive OPCs are widely distributed in the brain. During cuprizone treatment OPCs increase, peak at 4.5 weeks, then decrease but remain still elevated compared to the controls by 6 weeks, in both CC and cortex (**Figure 9**, Gudi et al., 2009). Parallel to myelin clearance in the CC, OPCs proliferate in the sub-ventricular zone and fornix then migrate towards demyelinating lesions and accumulate in CC (Mason et al., 2000a). Two transcription factors are important for the fate of oligodendrocytes: Olig1 and Olig2. They are expressed in both myelinating oligodendrocytes and OPCs in the developing CNS. Olig2 is necessary for the specification of oligodendrocytes during vertebrate embryogenesis. Olig1 has a reparative role and is involved in oligodendrocyte differentiation and consequent remyelination in the context of white matter injury (Arnett et al., 2004). Local and migrated OPCs terminally differentiate and repair myelin sheaths at the site of lesions. During remyelination, new mature oligodendrocytes are generated, mRNA levels of myelin genes return to normal and new remyelinated axons can be detected (**Figure 9**, Mason et al., 2000a; Gudi et al, 2009).

Remyelination starts also if the cuprizone treatment is not discontinued. In this case, the new generated oligodendrocytes are damaged again (Matsushima and Morell, 2001).

Chronic exposure to cuprizone (more than 12 weeks) induces apoptosis in new generated oligodendrocytes and decrease of OPC pool. However, transplanted adult OPCs could remyelinate the chronically demyelinated axons inside the lesions (Mason et al., 2004).

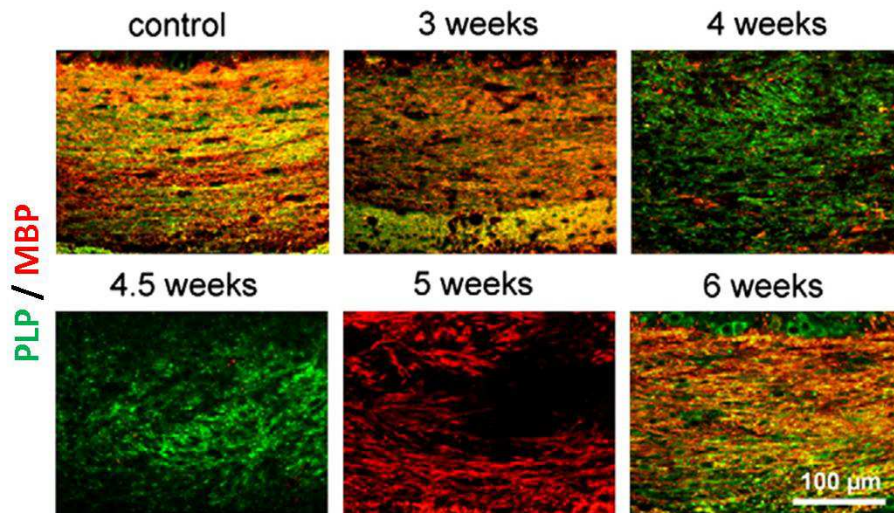


Figure 10. MBP/PLP double (MBP in red, PLP in green) immunofluorescence stained coronal sections of the medial CC during demyelination. Degradation of MBP occurred after 4 and 4.5 weeks of cuprizone treatment, followed by a recovery at week 5. Note, PLP degradation occurred late at week 5 with re-expression at week 5.5 of cuprizone diet (Gudi V, Moharreggh-Khiabani D, Skripuletz T, Koutsoudaki PN, Kotsiari A, Skuljec J, Trebst C, Stangel M. Regional differences between grey and white matter in cuprizone induced demyelination. Brain Res. 2009 Aug 4;1283:127-38).

- Microglia and macrophages

Microglia population is genetically distinct from macrophage population, even if sharing the same myeloid background. Microglia are CNS resident immune-competent cells and originate from erythromyeloid precursors in embryonic yolk sac (Alliot et al., 1999). In contrast macrophages take origin from bone marrow myeloid hematopoietic stem cells that differentiate into blood circulating monocytes (Ousman and Kubes, 2012). Under physiological condition, macrophages do not participate to microglia pool, but during CNS injury they may infiltrate in CNS parenchyma and accumulate at the lesion site, contributing to both tissue damage and repair.

Cuprizone exposure activates microglia and macrophages already after 2 weeks even before demyelination events take place (**Figure 9**, Hiremath et al., 1998). At week 3, the ionized calcium-binding adapter molecule 1 (IBA-1) / lectin ricinus communis agglutinin-1 (RCA-1)-positive microglia proliferate both in the CC and in the cortex. During weeks 5-6 of cuprizone treatment, activated microglia clear myelin debris and when demyelination events are complete, a small pool of activated microglial cells still persist in chronic lesions (Mason et al., 2004). Stronger microgliosis is more evident in white matter compared to gray matter, maybe due to higher myelin content/debris (Gudi et al., 2009). There is an unsolved debate about the detrimental or beneficial role of microglia and macrophages in experimental models of MS (Rawji and Yong, 2013). These cells may be polarized by microenvironment to differentiate into two different subsets. M1 polarized macrophages show microbicidal properties, through Th1

lymphocyte responses, while M2 activated macrophages display endocytic clearance capacities and express anti-inflammatory molecules promoting tissue repair, through Th2 lymphocyte responses (Martinez et al., 2008). In cuprizone model, microglia display an intermediate M1/M2 phenotype. Indeed, microglia play different roles: phagocyte myelin debris and apoptotic cells during demyelination; activate and recruit endogenous OPCs to the lesion site and deliver trophic support during remyelination (Olah et al., 2012). In particular, during demyelination microglia phenotype is characterized by CD11b⁺/CD45^{low} surface marker expression, together with several phagocytic receptors (such as Triggering Receptor Expressed on Myeloid cells 2b, Trem2b) and MHC class II molecule upregulation, especially at the peak of demyelination, promoting myelin debris phagocytosis. In a later phase, production of TNF α , Fibroblast Growth Factor 2 (FGF-2) and Insulin-like Growth factor 1 (IGF-1) generates a microenvironment suitable for remyelination (Voss et al., 2012). Microglia activation promotes T lymphocytes recruitment but these cells are not activated and do not actively infiltrate the lesions, remaining confined to perivascular cuffs (Remington et al., 2007). Moreover, a subset of CD11b⁺/CD45^{high} cells, which are speculated to be recruited peripheral macrophages, are found in the CC of cuprizone-treated mice starting from 2 weeks of cuprizone exposure and sustain microglia resident CNS population (Mc Mahon et al., 2002). Nevertheless, since there is no clear evidence of BBB leakage, it is likely that the cells are M1-type activated microglia, instead of peripheral macrophages.

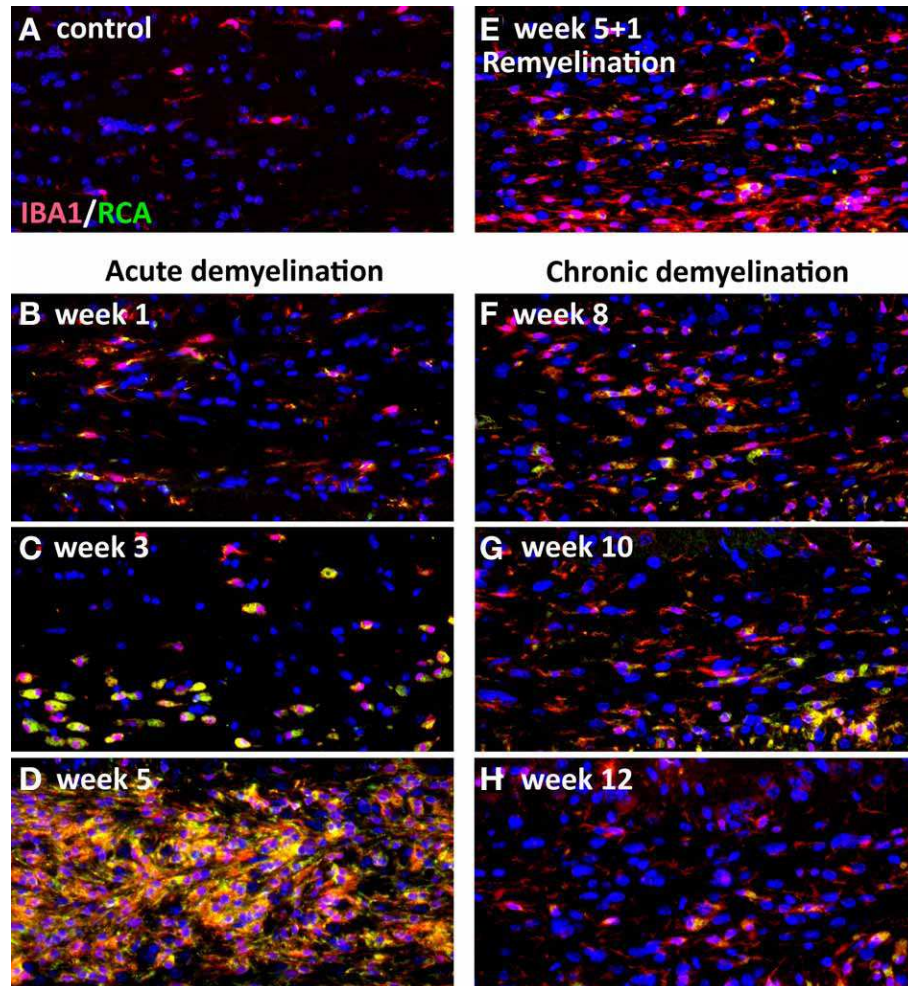


Figure 11. IBA-1/RCA double (IBA-1 general microglia marker in red, RCA activated microglia marker in green) immunofluorescence stained coronal sections of the medial CC during acute (B, C, D) and chronic (F, G, H) demyelination and remyelination (E) compared to control (A) (from Gudi V, Gingele S, Skripuletz T, Stangel M. Glial response during cuprizone-induced de- and remyelination in the CNS: lessons learned. *Front Cell Neurosci.* 2014 Mar 13;8:73).

- Astrocytes

Astrocytes are the most abundant cells in the adult CNS. Under physiological condition, astrocytes support maintenance and development of myelin through expression of cytokines and chemokines. In response to demyelination, astrocytes play a dual role both beneficial and detrimental on myelin status (Kipp et al., 2011; Barnett and Linington, 2013).

Cuprizone diet causes enlargement and hyperplasia of astroglia, as reported by Carlton in 1966 (Carlton, 1966). In particular, during the first 2 weeks of cuprizone administration, astrocytes show morphological changes and become hypertrophic with thick processes. Starting from week 3, the number of astrocytes greatly increases and peaks at maximal demyelination (**Figure 9**; Hiremath et al., 1998; Gudi et al., 2009). The number of Glial Fibrillary Acidic Protein (GFAP)-positive astrocytes is similar during acute and chronic demyelination (**Figure 12**, Kipp et al., 2011). Interestingly, microglia reactivity diminishes after early demyelination, instead astrocyte activation persists throughout demyelination and during the first weeks of recovery period (Hibbits et al., 2012). Skripuletz and colleagues demonstrated that transgenic mice (in which reactive astrocytes are ablated) fed with cuprizone show a reduced activation of microglia at the demyelinating lesions and a reduced amount of mature oligodendrocytes during remyelination (Skripuletz et al., 2013). Their results point out a key role for astrocytes in interacting with other cell subtypes supporting remyelination.

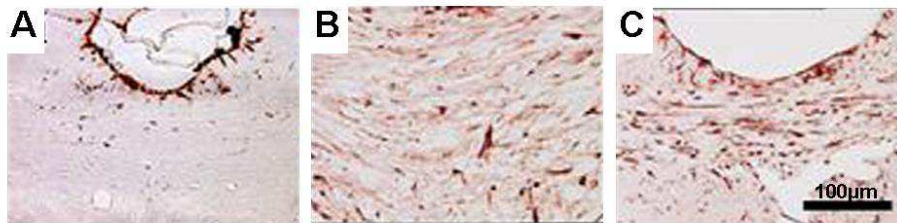


Figure 12. GFAP immunohistochemistry stained coronal sections of the medial CC during acute (B) and chronic (C), compared to control (A) (modified from Kipp M, Gingele S, Pott F, Clarner T, van der Valk P, Denecke B, Gan L, Siffrin V, Zipp F, Dreher W, Baumgartner W, Pfeifenbring S, Godbout R, Amor S, Beyer C. BLBP-expression in astrocytes during experimental demyelination and in human multiple sclerosis lesions. *Brain Behav Immun.* 2011 Nov;25(8):1554-68.).

- Neurons

Neurons are the main players in the transmission of information through electrical and chemical signals. Cuprizone administration does not attack directly neurons, but it damages oligodendrocytes that become unable to support myelin sheaths. Demyelinated axons are prone to be preferentially attacked by immune cells and inflammatory mediators (i.e. nitric oxide; Zendedel et al., 2013). Axonal damage is already observed after 5 weeks of cuprizone exposure, when nearly complete demyelination of axons is shown through electron microscopy (**Figure 13**, Skripuletz et al., 2013). Irvine and Blakemore reported that remyelination was able to protect axons from demyelination-associated axon degeneration and to restore axonal conduction properties (Irvine and Blakemore, 2008). On the contrary, Linder and colleagues demonstrated that axonal injury occurs during chronic demyelination, despite sufficient remyelination events take place (Lindner et al., 2009). This is probably due to a slow axonal degeneration that still continues at low levels and accumulates, despite complete remyelination (Zendedel et al., 2013). It has been demonstrated that a critical time window exists for restoring axonal conduction to normal level: 1.5 week of cuprizone diet causes minimal axonal loss, completely recovered after cuprizone withdrawal (Crawford et al., 2009). In addition to the duration of exposure, the age of animals is another critical factor in neuronal damage. Indeed, aged C57BL/6 mice (6-7 months) are more prone to axonal degeneration associated with primary demyelination than young adult mice (8-10 weeks; Irvine and Blakemore, 2006).

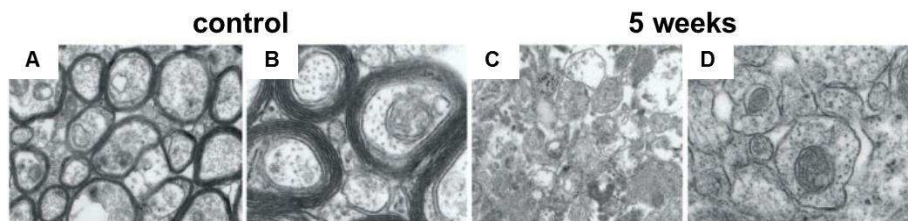


Figure 13. Ultrastructural analysis showed demyelinated axons in the CC of cuprizone 5 week treated mice (C, D) compared control mice (A, B) (modified from Skripuletz T, Hackstette D, Bauer K, Gudi V, Pul R, Voss E, Berger K, Kipp M, Baumgärtner W, Stangel M. Astrocytes regulate myelin clearance through recruitment of microglia during cuprizone-induced demyelination. *Brain*. 2013 Jan;136(Pt 1):147-67.).

1.3.5 Crosstalk between glia

Cytokines, chemokines, pro-inflammatory molecules and growth factors promote demyelination and modulate remyelination in cuprizone experimental model. It is essential to increase the knowledge on the roles played by that these molecules in the remyelination process in order to develop new successful therapies.

- Cytokines and pro-inflammatory molecules

In the cuprizone model inflammatory response is supported by activated microglia, astrogliosis and macrophages infiltration (Hiremath et al., 1998). Different factors expressed or secreted by these cells seem to play an important role not only in demyelination but also in remyelination. It appears that a controlled inflammatory response is necessary for myelin debris removal, oligodendrocyte proliferation and finally remyelination (Voss et al., 2012).

TNF- α is a pro-inflammatory cytokine, playing an important role in cuprizone model. It is usually involved in myelin damage and disease progression, but it also enhances remyelination mediated through the TNF receptor 2. The production of the TNF- α is elevated during demyelination. Arnett and colleagues demonstrated that mice lacking TNF- α are characterized by reduced oligodendrocyte loss and significant delay in remyelination, associated with reduction of proliferating OPCs. The same events are observable in mice lacking TNF receptor 2 (Arnett et al., 2001; Voss et al., 2012).

Another pro-inflammatory cytokine, that can play both a beneficial and a harmful role in cuprizone model, is IFN- γ . Gao and colleagues

described the IFN- γ action in MBP/IFN- γ transgenic mice (expressing low levels of IFN- γ under the transcriptional control of the MBP promoter) fed with cuprizone. Minimal demyelination and reduced oligodendrocyte death were detected in these mice, sustaining a protective role for IFN- γ (Gao et al., 2000). On the contrary, Maña and colleagues demonstrated a deleterious role of IFN- γ in cuprizone model. Indeed, they observed reduced demyelination and accelerated remyelination in mice lacking the binding chain of IFN- γ receptor (Maña et al., 2006). Similar discrepancies in the dichotomy between beneficial and harmful roles of IFN- γ were also observed in other MS models, such as EAE (Lin and Lin, 2010; Balabanov et al., 2007; Mangalam et al., 2014).

Probably, IFN- γ and other pro-inflammatory cytokines can play both a beneficial and harmful role, subordinate to their quantitative and their temporal expression (Gudi et al., 2014).

- Growth factors

In the CNS, growth factors are important to the development and maintenance of the structures, but they also play a powerful role in repair priming. In cuprizone model, several growth factors such as Fibroblast Growth Factor 2 (FGF-2) and Insulin-like Growth factor 1 (IGF-1), are strongly regulated during demyelination and remyelination processes (Gudi et al., 2011).

In particular, different temporal patterns of FGF-2 transcript expression characterize the CC and the cortex in cuprizone model. FGF-2 supports OPC proliferation and inhibits progenitor

differentiation (Bansal and Pfeiffer, 1997). FGF-2 is upregulated in both CC and cortex, in correspondence with the demyelination peak (4.5 weeks of cuprizone treatment). Instead, during remyelination, FGF-2 is still elevated in the CC but decreases in the cortex, suggesting differences in regulation of demyelination in white and grey matters (Gudi et al., 2011). The absence of FGF-2 (FGF-2 knock-out mice) allows faster differentiation of oligodendrocytes after demyelination and inducing increased oligodendrocyte density compared to control mice (Armstrong et al., 2002).

IGF-1 is another growth factor playing an important role in myelination. It promotes oligodendrocyte differentiation and increases the synthesis and accumulation of myelin (Mozell and McMorris, 1991). IGF-1 expression is similar in both CC and cortex and is characterized by a gradual increase during demyelination followed by a gradual decrease during remyelination (Gudi et al., 2011). The CC is demyelinated after 3 weeks of cuprizone treatment and the recovery is almost complete after 5 weeks of continuous exposure, in transgenic mice (that express IGF-1 under the transcriptional control of metallothionein 1 promoter). This faster recovery is due to the inhibition of mature oligodendrocytes apoptosis, preventing OPCs accumulation (Mason et al., 2000b).

- Chemokines

In the last years, the role of chemokines and their signaling has emerged as an important aspect in CNS homeostasis and plasticity. Recent studies have analysed the ability of chemokine receptor

CXCR3 and the corresponding ligands CXCL9, CXCL10, CXCL11 in modulating glia functions (Müller et al., 2007 and Krauthausen et al., 2014). In cuprizone model, CXCR3 signalling is directly involved in glia activation. Indeed, CXCR3 deficient mice treated with cuprizone show a reduction in microglia and astrocytes proliferation and activation, without significant differences in demyelination compared to wild-type mice (Krauthausen et al., 2014).

Another chemokine receptor, CXCR4, and its corresponding ligand, CXCL12, play a role in oligodendrocyte differentiation. During cuprizone demyelination, CXCL12 is significantly upregulated and promotes OPC differentiation via CXCR4, which is essential for efficient remyelination (Patel et al., 2012).

1.4.1 Magnetic resonance imaging

Longitudinal follow-up serves a fundamental purpose in clinical practice and research in MS, as it allows the evaluation of response to pharmaceutical intervention; in the case of animal models, it also allows characterizing the natural evolution of the model and comparing it with the idiopathic form of the disease (Comi et al., 2001).

At present, in clinical trials of MS therapeutics strong emphasis is still placed on the volumetric aspect of lesion load, i.e. on the number and overall volume of hyperintense areas. While this remains an important endpoint, there is increasing interest for the damage of the so-called normal-appearing white matter, which may be important in determining clinical condition and outcome. As a consequence, nowadays there is a broad interest in those imaging modalities that yield quantitative, parametric maps and, as such, support regional measurements independently of the presence and location of “discrete” hyperintense lesions (Filippi et al., 2011).

- Conventional magnetic resonance imaging

Conventional MRI is a sensitive tool to detect myelin status non-invasively and plays an important role in MS diagnosis. Nevertheless, MRI correlates poorly with clinical findings (Filippi et al., 1995). Different plaques in MS subtypes might be characterized by oedema, inflammatory infiltrates, glial hyperplasia and myelin sheath thinning. However, potential abnormalities, beyond the visible MRI lesions, exist in the normal appearing white (NAWM) and grey matter (Miller

et al., 2003). Conventional MRI is especially sensitive to focal white matter lesions and atrophy (Miller et al., 2014) and the imaging sequences commonly used in MS are the following:

- fast spin echo T2-weighted or proton density-weighted imaging, for detecting white matter plaque hyperintensity (Filippi et al., 2011);
- fluid-attenuated inversion recovery (FLAIR), useful to detect periventricular white matter lesions, suppressing cerebro-spinal fluid (**Figure 14**; Miller et al., 2014);
- non-contrast T1-weighted imaging, which detects white matter plaque hypointensity (**Figure 14**; Filippi et al., 2011);
- diffusion-weighted imaging shows diffusion restriction of water molecules associated with white matter lesions and acute inflammation (Miller et al., 2014);
- contrast (i.e. gadolinium)-enhanced T1-weighted imaging (Filippi et al., 2011).

In order to increase the sensitivity of MRI for early MS diagnosis, magnets with high-field (>3.0 Tesla) and ultrahigh-field (>7.0 Tesla) strengths are now used, pulsed sequences have been improved and new contrast agents are under development (Miller et al., 2014).

- Non-conventional magnetic resonance imaging

Conventional MRI plays an important role in the study of MS, however the obtained measures might be affected by secondary aspects of the disease, such as oedema, inflammation, demyelination and axonal loss. Indeed, all these pathological events are associated with increased water content, thus they might be indistinguishable on

conventional MRI images, which do not provide a specific direct measure of myelin integrity (Comi et al., 2001; Ge, 2006). To better characterize the MS plaques and monitor the subtle but pervasive pathological changes occurring in NAWM, non-conventional MRI techniques have emerged (Miller et al., 2014); the most commonly used are the following:

- magnetization transfer imaging (MTI), which evaluates damage affecting the “macromolecular pool”, which primarily consists of myelin (this technique is explained in detail in the following paragraphs);
- diffusion tensor imaging (DTI), which measures alterations in the Brownian motion of water molecules that are influenced by diffusion barriers such as cell membranes and macromolecular structures (**Figure 14**; Ingles and Bester, 2010);
- magnetic resonance proton spectroscopy (MRPS), which detects metabolite abnormalities in both white and gray matter (Chard et al., 2002);
- functional magnetic resonance imaging (fMRI), which reveals alteration in cortical activation (Miller et al., 2014).

Non-conventional MRI techniques are helpful to characterize structural, metabolic and functional changes and to more accurately link newly forming lesions with emerging MS symptoms. Although not currently considered as essential criteria for diagnosis, non-conventional MRI might trace, and more importantly predict, the dissemination of lesions in time and space (Miller et al., 2014).

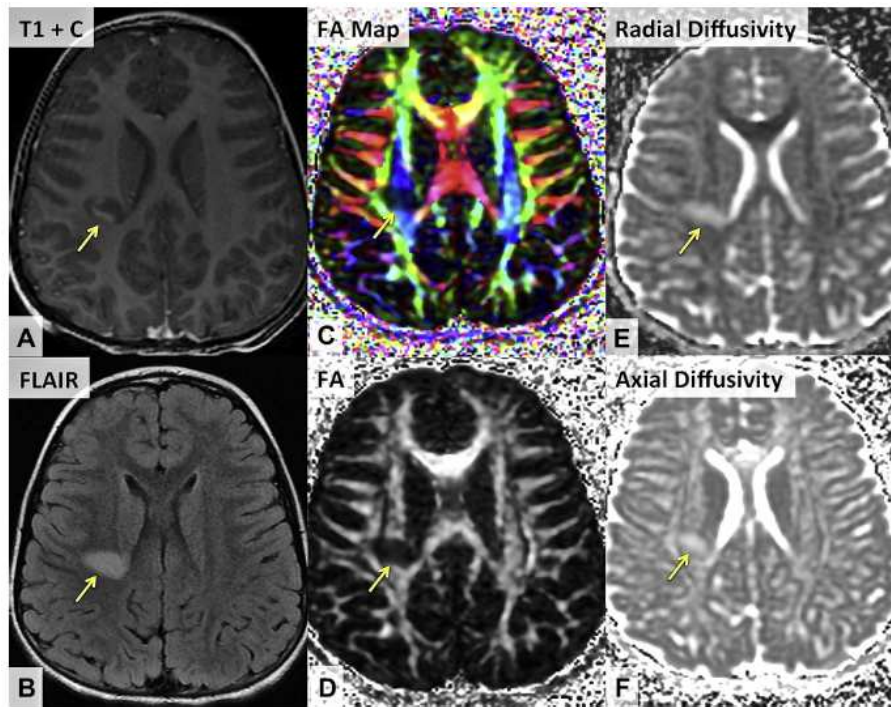


Figure 14. Conventional MRI shows an enhancing focal white matter lesion in the right centrum semiovale on both enhanced T1-weighted and FLAIR-weighted imaging (arrows in A and B). On diffusion tensor imaging (non-conventional MRI technique), the lesion shows a reduction in fractional anisotropy, FA (arrows in C and D), and an increase in diffusivity (arrows in E and F; from Miller TR, Mohan S, Choudhri AF, Gandhi D, Jindal G. Advances in multiple sclerosis and its variants: conventional and newer imaging techniques. Radiol Clin North Am. 2014 Mar;52(2):321-36).

1.4.2 Magnetization transfer imaging

In 1989, Wolff and Balaban introduced MTI as a useful MRI technique extending the capabilities of conventional MRI. By virtue of its high physical specificity, MTI increases tissue contrasts allowing more specific tissue characterization (Wolff and Balaban, 1989).

Measures obtained through this technique can be analyzed employing a two-pool model, representing hydrogen nuclei (protons) in biological tissue in different molecular environments: “free water”, i.e. ^1H nuclei that are in liquid or near-liquid media and as such mobile, and “bound macromolecular (i.e. myelin)” ^1H nuclei, which are in semi-solid state and, as such, relatively immobile. The longitudinal relaxation times (T_1) of these two pools are quite similar, however their transversal relaxation times (T_2) are significantly different. In particular, the T_2 s of bound macromolecular ^1H nuclei are so short that they are invisible using standard MRI equipment, because signal decays before readout is effectively possible. Owing to its molecular heterogeneity, this pool has a broad resonance line. On the contrary, T_2 of free water ^1H nuclei is quite long and this pool is characterized by a narrow resonance line, allowing effective imaging (**Figure 15**; Wolff and Balaban, 1989; Horsfield, 2005).

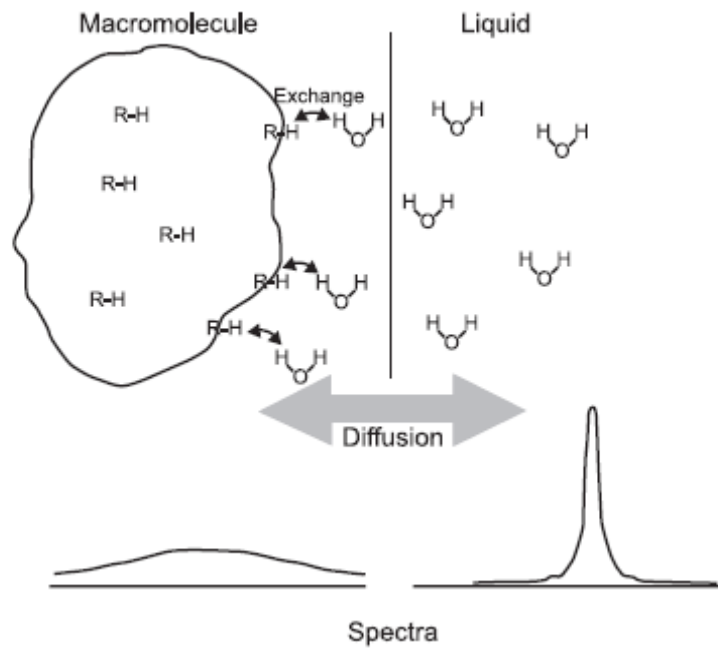


Figure 15. Representation of a macromolecule that has functional groups (R) with bound hydrogen atoms (H) that are available to exchange with those in water (Horsfield MA. Magnetization transfer imaging in multiple sclerosis. *J Neuroimaging*. 2005;15(4 Suppl):58S-67S).

The key notion of magnetization-transfer imaging is that the two pools are magnetically coupled and it is therefore possible to deduce the properties of the macromolecular pool by studying the transfer of magnetization between the pools. There is a continuous “exchange” of magnetization by ^1H nuclei characterized by different chemical environments, which can, at a statistical level, influence each other’s spin states (the proton magnetization state measured by MRI). Applying a radio frequency (RF) pulse with sufficient power, characterized by a frequency far from the central narrow resonance line of the water pool, can be used as a method to saturate ^1H nuclei in the bound pool, while having little direct effect on water protons (**Figure 16**, Wolff and Balaban, 1989; Horsfield, 2005). The RF power saturates the spins of macromolecular pool (the up and down spins are equalized) and the saturated macromolecular magnetization is transferred to the water pool. This event causes attenuation in the water signal intensity (referred to as Magnetization Transfer Ratio, MTR) detectable via MRI scanning (**Figure 16**, Wolff and Balaban, 1989; Horsfield, 2005).

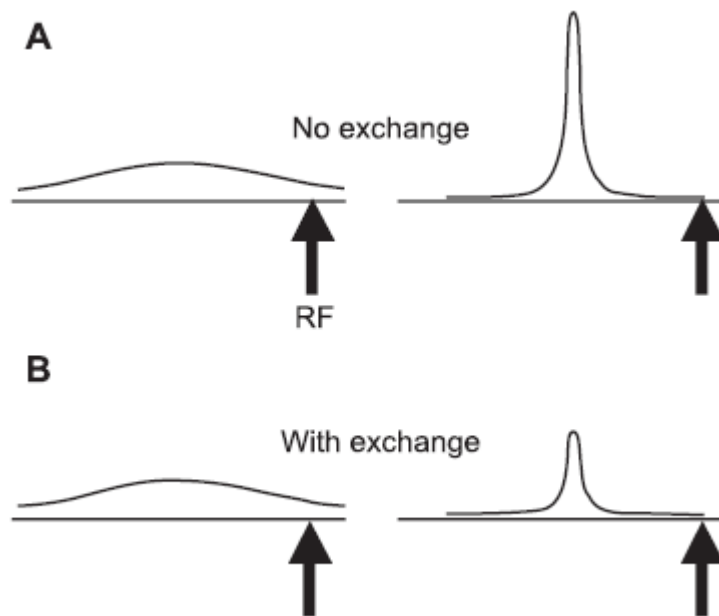


Figure 16. A radio frequency (RF) pulse, has no effect on the observable fluid spectrum if the fluid protons are not exchanging magnetization with the macromolecular protons (A). Whether there is exchange (B) the saturated macromolecular magnetization is transferred to the fluid, resulting in a reduction of the amplitude of the signal (Horsfield MA. Magnetization transfer imaging in multiple sclerosis. *J Neuroimaging*. 2005;15(4 Suppl):58S-67S).

- Macromolecular pool size ratio and bound proton fraction

In order to better characterize this model, Sled and Pike introduced an index, the macromolecular pool size ratio (F), which is hypothesized to directly correlate with the myelin content (that is the macromolecular pool of CNS; Sled and Pike, 2001):

$$F = \frac{{}^1\text{H bound pool}}{{}^1\text{H free pool}}$$

Another parameter usually defined is the bound proton fraction (f) that represents the percentage of proton in the macromolecular pool and corresponding to:

$$f = \frac{F}{F + 1}$$

The two parameters provide coincident information and, for typical measurements, are numerically very similar.

- Magnetization transfer ratio

In 1992, for the first time, Dousset and colleague used MTI to analyse the CNS tissue of EAE mice (Dousset et al., 1992). They defined the magnetization transfer ratio (MTR) as:

$$\text{MTR} = \frac{(M_0 - M_s)}{M_0} \cdot 100\%$$

Where:

- M_0 = proton density weighted signal measured in the absence of saturation (non-MT image);
- M_s = proton density weighted signal measured in the presence of saturation applied to the bound proton pool (MT image).

MTR is the percentage reduction in the water signal when the saturating pulse is applied (Barker et al., 1996). MTR values close to zero mean that magnetization transfer is absent, so the water spectrum is the same in absence and presence of saturating pulse (Horsfield, 2005). Free water and cerebro-spinal fluid have MTR values close to 0, white matter is characterized by high MTR values and grey matter displays intermediate values (Tofts, 2003).

- Ramani's signal equation and fitting

Starting from the two-pool Bloch model, in the 1993 Henkelman and colleagues studied agar gel model by systematically varying offset frequencies, amplitude of the RF irradiation and gel concentration. Their results demonstrated that a quantitative interpretation of magnetization transfer (qMT) is possible and the water pool is modeled with a Lorentzian line shape (Henkelman et al., 1993).

In 2002, Ramani developed a mathematical method to estimate MT model parameters by qMT and this is the rewritten Henkelman-Ramani expression (Ramani et al., 2002; Cercignani and Barker, 2008):

$$\begin{aligned}
 SI(\omega_1, \Delta f) &= \frac{M_0 \left(R_B \left[\frac{RM_0^B}{R_A} \right] + R_{RFB}(\omega_{1CWPE}, \Delta f) + R_B + \frac{RM_0^B}{F} \right)}{\left[\frac{RM_0^B}{R_A} \right] (R_B + R_{RFB}(\omega_{1CWPE}, \Delta f))} \\
 &\quad + \left(1 + \left[\frac{\omega_{1CWPE}}{2\pi\Delta f} \right]^2 \left[\frac{1}{R_A T_2^A} \right] \right) \left(R_{RFB}(\omega_{1CWPE}, \Delta f) + R_B + \frac{RM_0^B}{F} \right)
 \end{aligned}$$

Where:

- A identifies the water-free pool;
- B identifies the macromolecular-bound pool;
- M_0 = number of protons;
- $F = \frac{M_0^B}{M_0^A}$;
- R_A and R_B = longitudinal relaxation times (T1 observed) of the two pools;
- T_2^A and T_2^B = transverse relaxation times (T2) of the two pools;
- RM_0^B = forward exchange rate (k_f), a time constant of the ^1H nuclei from water pool to macromolecular pool;
- $\omega_{1CWPE} = \gamma\sqrt{P_{SAT}}$; P_{SAT} is the mean square saturating field and CWPE means continuous wave power equivalent (a train of pulses with spacing minus of T1, that mimics a constant irradiation).

In this model seven parameters are (M_0 , R_A , R_B , RM_0^B , F , T_2^A and T_2^B) free and cannot be uniquely determined. They are reduced to five (R_A , R_B are removed), which can be estimated by fitting the equation (Cercignani and Barker, 2008).

The biological interpretation of RM_0^B and T_2^B is less clear than that of F , f and MTR which are direct linked to myelin status. There is some evidence that RM_0^B may be related to metabolic changes, and that T_2^B , which generally shows small and inconsistent changes in pathology, may be influenced by fibre orientation (Cercignani et al., 2009).

- Factors altering measured magnetization transfer values

The MT effect depends on several factors. Firstly, the properties of tissues such as macromolecular concentration, the sites available to exchange magnetization and hydration, might alter MT measured parameters. These are intrinsic characteristics of the biological tissue examined and no intervention to minimize such factors is possible (Horsfield 2005; Tofts 2003). Secondly, the features of pulse sequence, in particular incorrect chose offset frequency and degree of saturation, T_1 -weighting effects, inhomogeneous B_1 , might alter MT measured values. It is important to balance the necessity to obtain good MT contrasted images with that of maintaining relatively short acquisition times (Horsfield 2005; Tofts 2003). Finally also adverse factors (i.e. image noise, patient movement) might alter MT images and should be partially minimized by choosing appropriate MRI sequences.

1.4.3 Quantitative magnetization transfer imaging in experimental models of demyelination

Quantitative Magnetization Transfer (qMT) produces a quantitative determination of the bound proton fraction referred to as f , or equivalently of the macromolecular pool size ratio F , and relate f and F to signal attenuation as a function of saturation pulse frequency offset and intensity, as well as T_1 relaxation time (Tofts, 2003).

In recent years, studies on experimental models of demyelination have investigated the correspondence between myelin status determined histologically, using conventional staining, immuno-staining and

electro-microscopy, and the imaging parameters MTR or f (and, equivalently, F) measured especially in ex-vivo conditions (Ou et al., 2009; Thiessen et al., 2013). Thiessen and colleagues compared qMT and diffusion tensor imaging measured ex-vivo with values obtained by ultrastructural examination of cuprizone mouse brain and found that f is the strongest indicator of myelin content (Thiessen et al., 2013). Ou and colleagues demonstrated ex-vivo that F is substantially reduced in the white matter of shiverer mice, that have nearly no myelin whilst keeping normal brain cytoarchitecture, compared to wild type controls (Ou et al., 2009). Their results confirm the link between these parameters (f and F) and myelin status, but ex vivo scans, following perfusion and complete tissue fixation, might not accurately represent in vivo imaging conditions (Ou et al., 2009; Thiessen et al., 2013).

To date few studies have investigated the correspondence between in vivo measures of MTR, f and F , and myelin status as measured by histology in experimental models of demyelination (Rausch et al., 2009; Aharoni et al., 2013; Fjær et al., 2013). Using a focal EAE rat model, Rausch and colleagues demonstrated that MT properties of the tissue closely correspond to demyelination (Rausch et al., 2009). Moreover, the correlation between MTR measures and histological values have been confirmed in two EAE model (the chronic MOG-induced and the relapsing-remitting PLP-induced) and have been applied to confirm in vivo the efficacy of glatiramer acetate treatment (Aharoni et al., 2013). Finally, first evidence is emerging also in the cuprizone model regarding the correlation between MTR values and progressive myelin loss in both CC and deep grey matter (Fjær et al.,

2013). All these promising studies need further confirmation, but underline the importance of qMT as useful tool in preclinical research, which might lead to reduce the number of needed experimental animals, otherwise sacrificed for histological analysis. Finally, qMT might soon represent a reliable technique also for clinical MS diagnosis.

1.5.1 Scope of the thesis

The aim of this study is to verify whether the qMT parameter, macromolecular pool size ratio (F), measured in vivo at 7.0 Tesla, correlates with ex vivo histological analysis. In the present work both demyelination and remyelination events are investigated. Moreover, two murine strains (C57BL/6 and SJL/J), characterized by different sensitivity to cuprizone intoxication, are used.

1.6.1 References

Acs P, Kipp M, Norkute A, Johann S, Clarner T, Braun A, Berente Z, Komoly S, Beyer C. 17beta-estradiol and progesterone prevent cuprizone provoked demyelination of corpus callosum in male mice. *Glia*. 2009 Jun;57(8):807-14.

Adzemovic MZ, Zeitelhofer M, Hochmeister S, Gustafsson SA, Jagodic M. Efficacy of vitamin D in treating multiple sclerosis-like neuroinflammation depends on developmental stage. *Exp Neurol*. 2013 Nov;249:39-48.

Aharoni R, Sasson E, Blumenfeld-Katzir T, Eilam R, Sela M, Assaf Y, Arnon R. Magnetic resonance imaging characterization of different experimental autoimmune encephalomyelitis models and the therapeutic effect of glatiramer acetate. *Exp Neurol*. 2013 Feb;240:130-44.

Alliot F, Godin I, Pessac B. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Brain Res Dev Brain Res*. 1999 Nov 18;117(2):145-52.

Apperson ML, Tian Y, Stamova B, Ander BP, Jickling GC, Agius MA, Sharp FR. Genome wide differences of gene expression associated with HLA-DRB1 genotype in multiple sclerosis: a pilot study. *J Neuroimmunol*. 2013 Apr 15;257(1-2):90-6.

Armstrong RC, Le TQ, Frost EE, Borke RC, Vana AC. Absence of fibroblast growth factor 2 promotes oligodendroglial repopulation of demyelinated white matter. *J Neurosci*. 2002 Oct 1;22(19):8574-85.

Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci*. 2001 Nov;4(11):1116-22.

Arnett HA, Fancy SP, Alberta JA, Zhao C, Plant SR, Kaing S, Raine CS, Rowitch DH, Franklin RJ, Stiles CD. bHLH transcription factor Olig1 is required to repair demyelinated lesions in the CNS. *Science*. 2004 Dec 17;306(5704):2111-5.

Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol*. 2007 Apr;61(4):288-99.

Bakker DA, Ludwin SK. Blood-brain barrier permeability during Cuprizone-induced demyelination. Implications for the pathogenesis of immune-mediated demyelinating diseases. *J Neurol Sci*. 1987 Apr;78(2):125-37.

Balabanov R, Strand K, Goswami R, McMahon E, Begolka W, Miller SD, Popko B. Interferon-gamma-oligodendrocyte interactions in the regulation of experimental autoimmune encephalomyelitis. *J Neurosci*. 2007 Feb 21;27(8):2013-24.

Bansal R, Pfeiffer SE. FGF-2 converts mature oligodendrocytes to a novel phenotype. *J Neurosci Res*. 1997 Oct 15;50(2):215-28.

Barker GJ, Tofts PS, Gass A. An interleaved sequence for accurate and reproducible clinical measurement of magnetization transfer ratio. *Magn Reson Imaging*. 1996;14(4):403-11.

Barnett MH, Prineas JW. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol*. 2004 Apr;55(4):458-68.

Barnett SC, Linington C. Myelination: do astrocytes play a role? *Neuroscientist*. 2013 Oct;19(5):442-50.

Carlton WW. Response of mice to the chelating agents sodium diethyldithiocarbamate, alpha-benzoinoxime, and biscyclohexanone oxaldihydrazone. *Toxicol Appl Pharmacol*. 1966 May;8(3):512-21.

Cercignani M, Barker GJ. A comparison between equations describing in vivo MT: the effects of noise and sequence parameters. *J Magn Reson*. 2008 Apr;191(2):171-83.

Cercignani M, Basile B, Spanò B, Comanducci G, Fasano F, Caltagirone C, Nocentini U, Bozzali M. Investigation of quantitative magnetisation transfer parameters of lesions and normal appearing white matter in multiple sclerosis. *NMR Biomed*. 2009; 22:646-53.

Charcot JM. Histologie de la sclerose en plaque. *Gaz HopParis* 1868; 41:554-5 557-8,566.

Chard DT, Griffin CM, McLean MA, Kapeller P, Kapoor R, Thompson AJ, Miller DH. Brain metabolite changes in cortical grey and normal-appearing white matter in clinically early relapsing-remitting multiple sclerosis. *Brain*. 2002 Oct;125(Pt 10):2342-52.

Comi G, Rovaris M, Leocani L, Martinelli V, Filippi M. Clinical and MRI assessment of brain damage in MS. *Neurol Sci*. 2001 Nov;22 Suppl 2:S123-7.

Connor JR, Menzies SL. Relationship of iron to oligodendrocytes and myelination. *Glia*. 1996 Jun;17(2):83-93.

Crawford DK, Mangiardi M, Xia X, López-Valdés HE, Tiwari-Woodruff SK. Functional recovery of callosal axons following demyelination: a critical window. *Neuroscience*. 2009 Dec 29;164(4):1407-21.

Dal Canto MC, Kim BS, Miller SD, Melvold RW. Theiler's Murine Encephalomyelitis Virus (TMEV)-Induced Demyelination: A Model for Human Multiple Sclerosis. *Methods*. 1996 Dec;10(3):453-61.

DeLuca LE, Pikor NB, O'Leary J, Galicia-Rosas G, Ward LA, Defreitas D, Finlay TM, Ousman SS, Osborne LR, Gommerman JL. Substrain differences reveal novel disease-modifying gene candidates

that alter the clinical course of a rodent model of multiple sclerosis. *J Immunol*. 2010 Mar 15;184(6):3174-85.

Dousset V, Grossman RI, Ramer KN, Schnall MD, Young LH, Gonzalez-Scarano F, Lavi E, Cohen JA. Experimental allergic encephalomyelitis and multiple sclerosis: lesion characterization with magnetization transfer imaging. *Radiology*. 1992 Feb;182(2):483-91.

Duncan ID, Kondo Y, Zhang SC. The myelin mutants as models to study myelin repair in the leukodystrophies. *Neurotherapeutics*. 2011 Oct;8(4):607-24.

Dwosh E, Guimond C, Duquette P, Sadovnick AD. The interaction of MS and pregnancy: a critical review. *Int MS J*. 2003 Jun;10(2):38-42.

Filippi M, Horsfield MA, Tofts PS, Barkhof F, Thompson AJ, Miller DH. Quantitative assessment of MRI lesion load in monitoring the evolution of multiple sclerosis. *Brain*. 1995 Dec;118 (Pt 6):1601-12.

Filippi M, Rocca MA, De Stefano N, Enzinger C, Fisher E, Horsfield MA, Inglese M, Pelletier D, Comi G. Magnetic resonance techniques in multiple sclerosis: the present and the future. *Arch Neurol*. 2011 Dec;68(12):1514-20.

Fjær S, Bø L, Lundervold A, Myhr KM, Pavlin T, Torkildsen O, Wergeland S. Deep gray matter demyelination detected by

magnetization transfer ratio in the cuprizone model. PLoS One 2013; 12: e84162.

Franco-Pons N, Torrente M, Colomina MT, Vilella E. Behavioral deficits in the cuprizone-induced murine model of demyelination/remyelination. Toxicol Lett. 2007 Mar 30;169(3):205-13.

Gao X, Gillig TA, Ye P, D'Ercole AJ, Matsushima GK, Popko B. Interferon-gamma protects against cuprizone-induced demyelination. Mol Cell Neurosci. 2000 Oct;16(4):338-49.

Ge Y. Multiple sclerosis: the role of MR imaging. AJNR Am J Neuroradiol. 2006; 27(6):1165-76.

Gudi V, Moharreggh-Khiabani D, Skripuletz T, Koutsoudaki PN, Kotsiari A, Skuljec J, Trebst C, Stangel M. Regional differences between grey and white matter in cuprizone induced demyelination. Brain Res. 2009 Aug 4;1283:127-38.

Gudi V, Škuljec J, Yildiz Ö, Frichert K, Skripuletz T, Moharreggh-Khiabani D, Voss E, Wissel K, Wolter S, Stangel M. Spatial and temporal profiles of growth factor expression during CNS demyelination reveal the dynamics of repair priming. PLoS One. 2011;6(7):e22623.

Gudi V, Gingele S, Skripuletz T, Stangel M. Glial response during cuprizone-induced de- and remyelination in the CNS: lessons learned. *Front Cell Neurosci.* 2014 Mar 13;8:73.

Hawkes CH, Macgregor AJ. Twin studies and the heritability of MS: a conclusion. *Mult Scler.* 2009 Jun;15(6):661-7.

Henkelman RM, Huang X, Xiang QS, Stanisz GJ, Swanson SD, Bronskill MJ. Quantitative interpretation of magnetization transfer. *Magn Reson Med.* 1993 Jun;29(6):759-66.

Hesse A, Wagner M, Held J, Brück W, Salinas-Riester G, Hao Z, Waisman A, Kuhlmann T. In toxic demyelination oligodendroglial cell death occurs early and is FAS independent. *Neurobiol Dis.* 2010 Feb;37(2):362-9.

Hibbits N, Yoshino J, Le TQ, Armstrong RC. Astrogliosis during acute and chronic cuprizone demyelination and implications for remyelination. *ASN Neuro.* 2012 Oct 30;4(6):393-408.

Hiremath MM, Saito Y, Knapp GW, Ting JP, Suzuki K, Matsushima GK. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. *J Neuroimmunol.* 1998 Dec 1;92(1-2):38-49.

Hiremath MM, Chen VS, Suzuki K, Ting JP, Matsushima GK. MHC class II exacerbates demyelination in vivo independently of T cells. *J Neuroimmunol.* 2008 Oct 15;203(1):23-32.

Horsfield MA. Magnetization transfer imaging in multiple sclerosis. *J Neuroimaging.* 2005;15(4 Suppl):58S-67S.

Inglese M, Bester M. Diffusion imaging in multiple sclerosis: research and clinical implications. *NMR Biomed.* 2010 Aug;23(7):865-72.

Irvine KA and Blakemore WF. Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. *J Neuroimmunol.* 2006 Jun;175(1-2):69-76.

Irvine KA, Blakemore WF. Remyelination protects axons from demyelination-associated axon degeneration. *Brain.* 2008 Jun;131(Pt 6):1464-77.

Kipp M, Gingele S, Pott F, Clarner T, van der Valk P, Denecke B, Gan L, Siffrin V, Zipp F, Dreher W, Baumgartner W, Pfeifenbring S, Godbout R, Amor S, Beyer C. BLBP-expression in astrocytes during experimental demyelination and in human multiple sclerosis lesions. *Brain Behav Immun.* 2011 Nov;25(8):1554-68.

Kojima K, Wekerle H, Lassmann H, Berger T, Linington C. Induction of experimental autoimmune encephalomyelitis by CD4⁺ T cells

specific for an astrocyte protein, S100 beta. *J Neural Transm Suppl.* 1997;49:43-51.

Kornek B, Storch MK, Weissert R, Wallstroem E, Stefferl A, Olsson T, Linington C, Schmidbauer M, Lassmann H. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am J Pathol.* 2000 Jul;157(1):267-76.

Krauthausen M, Saxe S, Zimmermann J, Emrich M, Heneka MT, Müller M. CXCR3 modulates glial accumulation and activation in cuprizone-induced demyelination of the central nervous system. *J Neuroinflammation.* 2014 Jun 16;11:109.

Kurtzke JF. Epidemiology and etiology of multiple sclerosis. *Phys Med Rehabil Clin N Am.* 2005 May;16(2):327-49.

Lassmann H. Multiple sclerosis: is there neurodegeneration independent from inflammation? *J Neurol Sci.* 2007 Aug 15;259(1-2):3-6.

Li J, Ghiani CA, Kim JY, Liu A, Sandoval J, DeVellis J, Casaccia-Bonnel P. Inhibition of p53 transcriptional activity: a potential target for future development of therapeutic strategies for primary demyelination. *J Neurosci.* 2008 Jun 11;28(24):6118-27.

Lin W, Lin Y. Interferon- γ inhibits central nervous system myelination through both STAT1-dependent and STAT1-independent pathways. *J Neurosci Res*. 2010 Sep;88(12):2569-77.

Lindner M, Fokuhl J, Linsmeier F, Trebst C, Stangel M. Chronic toxic demyelination in the central nervous system leads to axonal damage despite remyelination. *Neurosci Lett*. 2009 Apr 3;453(2):120-5.

Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology*. 1996 Apr;46(4):907-11.

Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sørensen PS, Thompson AJ, Wolinsky JS, Balcer LJ, Banwell B, Barkhof F, Bebo B Jr, Calabresi PA, Clanet M, Comi G, Fox RJ, Freedman MS, Goodman AD, Inglese M, Kappos L, Kieseier BC, Lincoln JA, Lubetzki C, Miller AE, Montalban X, O'Connor PW, Petkau J, Pozzilli C, Rudick RA, Sormani MP, Stüve O, Waubant E, Polman CH. Defining the clinical course of multiple sclerosis: The 2013 revisions. *Neurology*. 2014 Jul 15;83(3):278-286.

Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol*. 2000 Jun;47(6):707-17.

Lucchinetti CF, Parisi J, Bruck W. The pathology of multiple sclerosis. *Neurol Clin.* 2005 Feb;23(1):77-105, vi.

Maña P, Liñares D, Fordham S, Staykova M, Willenborg D. Deleterious role of IFN γ in a toxic model of central nervous system demyelination. *Am J Pathol.* 2006 May;168(5):1464-73.

Mangalam AK, Luo N, Luckey D, Papke L, Hubbard A, Wussow A, Smart M, Giri S, Rodriguez M, David C. Absence of IFN- γ Increases Brain Pathology in Experimental Autoimmune Encephalomyelitis-Susceptible DRB1*0301.DQ8 HLA Transgenic Mice through Secretion of Proinflammatory Cytokine IL-17 and Induction of Pathogenic Monocytes/Microglia into the Central Nervous System. *J Immunol.* 2014 Oct 22. pii: 1302008.

Marrie RA. Environmental risk factors in multiple sclerosis aetiology. *Lancet Neurol.* 2004 Dec;3(12):709-18.

Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci.* 2008 Jan 1;13:453-61.

Mason JL, Jones JJ, Taniike M, Morell P, Suzuki K, Matsushima GK. Mature oligodendrocyte apoptosis precedes IGF-1 production and oligodendrocyte progenitor accumulation and differentiation during demyelination/remyelination. *J Neurosci Res.* 2000a Aug 1;61(3):251-62.

Mason JL, Ye P, Suzuki K, D'Ercole AJ, Matsushima GK. Insulin-like growth factor-1 inhibits mature oligodendrocyte apoptosis during primary demyelination. *J Neurosci*. 2000b Aug 1;20(15):5703-8.

Mason JL, Toews A, Hostettler JD, Morell P, Suzuki K, Goldman JE, Matsushima GK. Oligodendrocytes and progenitors become progressively depleted within chronically demyelinated lesions. *Am J Pathol*. 2004 May;164(5):1673-82.

Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol*. 2001 Jan;11(1):107-16.

McMahon EJ, Suzuki K, Matsushima GK. Peripheral macrophage recruitment in cuprizone-induced CNS demyelination despite an intact blood-brain barrier. *J Neuroimmunol*. 2002 Sep;130(1-2):32-45.

Mecha M, Carrillo-Salinas FJ, Mestre L, Feliú A, Guaza C. Viral models of multiple sclerosis: neurodegeneration and demyelination in mice infected with Theiler's virus. *Prog Neurobiol*. 2013 Feb-Mar;101-102:46-64.

Mikoshiba K, Okano H, Aruga J, Tamura T, Miura M, Ikenaka K, Nakagawa T. Chimeric and molecular genetic analysis of myelin-deficient (shiverer and mld) mutant mice. *Ann N Y Acad Sci*. 1990;605:166-82.

Miller DH, Thompson AJ, Filippi M. Magnetic resonance studies of abnormalities in the normal appearing white matter and grey matter in multiple sclerosis. *J Neurol*. 2003 Dec;250(12):1407-19.

Miller SD, Turley DM, Podojil JR. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat Rev Immunol*. 2007 Sep;7(9):665-77.

Miller TR, Mohan S, Choudhri AF, Gandhi D, Jindal G. Advances in multiple sclerosis and its variants: conventional and newer imaging techniques. *Radiol Clin North Am*. 2014 Mar;52(2):321-36.

Milo R, Kahana E. Multiple sclerosis: geoepidemiology, genetics and the environment. *Autoimmun Rev*. 2010 Mar;9(5):A387-94.

Milo R, Miller A. Revised diagnostic criteria of multiple sclerosis. *Autoimmun Rev*. 2014 Apr-May;13(4-5):518-24.

Min Y, Kristiansen K, Boggs JM, Husted C, Zasadzinski JA, Israelachvili J. Interaction forces and adhesion of supported myelin lipid bilayers modulated by myelin basic protein. *Proc Natl Acad Sci U S A*. 2009 Mar 3;106(9):3154-9.

Morell P, Barrett CV, Mason JL, Toews AD, Hostettler JD, Knapp GW, Matsushima GK. Gene expression in brain during cuprizone-

induced demyelination and remyelination. *Mol Cell Neurosci*. 1998 Nov;12(4-5):220-7.

Mozell RL, McMorris FA. Insulin-like growth factor I stimulates oligodendrocyte development and myelination in rat brain aggregate cultures. *J Neurosci Res*. 1991 Oct;30(2):382-90.

Müller M, Carter SL, Hofer MJ, Manders P, Getts DR, Getts MT, Dreykluft A, Lu B, Gerard C, King NJ, Campbell IL. CXCR3 signaling reduces the severity of experimental autoimmune encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system. *J Immunol*. 2007 Sep 1;179(5):2774-86.

Norkute A, Hieble A, Braun A, Johann S, Clarner T, Baumgartner W, Beyer C, Kipp M. Cuprizone treatment induces demyelination and astrogliosis in the mouse hippocampus. *J Neurosci Res*. 2009 May 1;87(6):1343-55.

Olah M, Amor S, Brouwer N, Vinet J, Eggen B, Biber K, Boddeke HW. Identification of a microglia phenotype supportive of remyelination. *Glia*. 2012 Feb;60(2):306-21.

Ou X, Sun SW, Liang HF, Song SK, Gochberg DF. The MT pool size ratio and the DTI radial diffusivity may reflect the myelination in shiverer and control mice. *NMR Biomed*. 2009; 22:480-7.

Ousman SS, Kubes P. Immune surveillance in the central nervous system. *Nat Neurosci*. 2012 Jul 26;15(8):1096-101.

Pasquini LA, Calatayud CA, Bertone Uña AL, Millet V, Pasquini JM, Soto EF. The neurotoxic effect of cuprizone on oligodendrocytes depends on the presence of pro-inflammatory cytokines secreted by microglia. *Neurochem Res*. 2007 Feb;32(2):279-92.

Patel JR, Williams JL, Muccigrosso MM, Liu L, Sun T, Rubin JB, Klein RS. Astrocyte TNFR2 is required for CXCL12-mediated regulation of oligodendrocyte progenitor proliferation and differentiation within the adult CNS. *Acta Neuropathol*. 2012 Dec;124(6):847-60.

Paz Soldán MM, Pirko I. Biogenesis and significance of central nervous system myelin. *Semin Neurol*. 2012 Feb;32(1):9-14.

Ramani A, Dalton C, Miller DH, Tofts PS, Barker GJ. Precise estimate of fundamental in-vivo MT parameters in human brain in clinically feasible times. *Magn Reson Imaging*. 2002 Dec;20(10):721-31.

Rausch M, Tofts P, Lervik P, Walmsley A, Mir A, Schubart A, Seabrook T. Characterization of white matter damage in animal models of multiple sclerosis by magnetization transfer ratio and quantitative mapping of the apparent bound proton fraction f. *MultScler*. 2009; 15(1):16-27.

Rawji KS, Yong VW. The benefits and detriments of macrophages/microglia in models of multiple sclerosis. *Clin Dev Immunol*. 2013;2013:948976.

Remington LT, Babcock AA, Zehntner SP, Owens T. Microglial recruitment, activation, and proliferation in response to primary demyelination. *Am J Pathol*. 2007 May;170(5):1713-24.

Rivers TM, Sprunt DH, Berry GP. Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. *J Exp Med*. 1933 Jun 30;58(1):39-53.

Rodriguez M, David CS. Demyelination induced by Theiler's virus: influence of the H-2 haplotype. *J Immunol*. 1985 Sep;135(3):2145-8.

Schluesener HJ, Wekerle H. Autoaggressive T lymphocyte lines recognizing the encephalitogenic region of myelin basic protein: in vitro selection from unprimed rat T lymphocyte populations. *J Immunol*. 1985 Nov;135(5):3128-33.

Simons M, Trotter J. Wrapping it up: the cell biology of myelination. *Curr Opin Neurobiol*. 2007 Oct;17(5):533-40. Epub 2007 Oct 17.

Skripuletz T, Hackstette D, Bauer K, Gudi V, Pul R, Voss E, Berger K, Kipp M, Baumgärtner W, Stangel M. Astrocytes regulate myelin

clearance through recruitment of microglia during cuprizone-induced demyelination. *Brain*. 2013 Jan;136(Pt 1):147-67.

Sled JG, Pike GB. Quantitative imaging of magnetization transfer exchange and relaxation properties in vivo using MRI. *Magn Reson Med*. 2001 Nov;46(5):923-31.

Sun D, Wekerle H. Ia-restricted encephalitogenic T lymphocytes mediating EAE lyse autoantigen-presenting astrocytes. *Nature*. 1986 Mar 6-12;320(6057):70-2.

Sun D, Whitaker JN, Huang Z, Liu D, Coleclough C, Wekerle H, Raine CS. Myelin antigen-specific CD8⁺ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol*. 2001 Jun 15;166(12):7579-87.

Suzuki K, Kikkawa Y. Status spongiosus of CNS and hepatic changes induced by cuprizone (biscyclohexanone oxalyldihydrazone). *Am J Pathol*. 1969 Feb;54(2):307-25.

Taylor LC, Gilmore W, Matsushima GK. SJL mice exposed to cuprizone intoxication reveal strain and gender pattern differences in demyelination. *Brain Pathol*. 2009 Jul;19(3):467-79.

Taylor LC, Gilmore W, Ting JP, Matsushima GK. Cuprizone induces similar demyelination in male and female C57BL/6 mice and results

in disruption of the estrous cycle. *J Neurosci Res.* 2010 Feb 1;88(2):391-402.

Theiler M. Spontaneous encephalomyelitis of mice, a new virus disease. *Exp Med.* 1937 Apr 30;65(5):705-19.

Thiessen JD, Zhang Y, Zhang H, Wang L, Buist R, Del Bigio MR, Kong J, Li XM, Martin M. Quantitative MRI and ultrastructural examination of the cuprizone mouse model of demyelination. *NMR Biomed.* 2013; 26(11):1562-81.

Tofts P. Quantitative MRI of the brain. Measuring changes caused by disease. Wiley. 2003.

Torkildsen O, Brunborg LA, Myhr KM, Bø L. The cuprizone model for demyelination. *Acta Neurol Scand Suppl.* 2008;188:72-6.

Tsunoda I, Fujinami RS. Inside-Out versus Outside-In models for virus induced demyelination: axonal damage triggering demyelination. *Springer Semin Immunopathol.* 2002;24(2):105-25.

Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol.* 2002 Feb;2(2):85-95.

Venturini G. Enzymic activities and sodium, potassium and copper concentrations in mouse brain and liver after cuprizone treatment in vivo. *J Neurochem.* 1973 Nov;21(5):1147-51.

Voss EV, Škuljec J, Gudi V, Skripuletz T, Pul R, Trebst C, Stangel M. Characterisation of microglia during de- and remyelination: can they create a repair promoting environment? *Neurobiol Dis.* 2012 Jan;45(1):519-28.

Wang H, Li C, Wang H, Mei F, Liu Z, Shen HY, Xiao L. Cuprizone-induced demyelination in mice: age-related vulnerability and exploratory behavior deficit. *Neurosci Bull.* 2013 Apr;29(2):251-9.

Waxman SG. Axonal conduction and injury in multiple sclerosis: the role of sodium channels. *Nat Rev Neurosci.* 2006 Dec;7(12):932-41.

Wolff SD, Balaban RS. Magnetization transfer contrast (MTC) and tissue water proton relaxation in vivo. *Magn Reson Med.* 1989 Apr;10(1):135-44.

World Health Organization: Atlas multiple sclerosis resources in the world 2008. Geneva: WHO Press; 2008.

Ye JN, Chen XS, Su L, Liu YL, Cai QY, Zhan XL, Xu Y, Zhao SF, Yao ZX. Progesterone alleviates neural behavioral deficits and demyelination with reduced degeneration of oligodendroglial cells in cuprizone-induced mice. *PLoS One.* 2013;8(1):e54590.

Zendedel A, Beyer C, Kipp M. Cuprizone-induced demyelination as a tool to study remyelination and axonal protection. *J Mol Neurosci*. 2013 Oct;51(2):567-72.

CHAPTER 2

In vivo quantitative magnetization transfer imaging correlates with histology during de- and re-myelination in cuprizone-treated mice

Paper Submitted to NMR in Biomedicine

Laura Turati¹, MSc; Marco Moscatelli¹, MD; Alfonso Mastropietro², PhD; Nicholas G Dowell³, PhD; Ileana Zucca², PhD; Alessandra Erbetta⁴, MD; Chiara Cordiglieri¹, PhD; Greta Brenna¹, MSc; Beatrice Bianchi¹, MSc; Renato Mantegazza¹, MD; Mara Cercignani³, PhD; Fulvio Baggi^{1#}, PhD; Ludovico Minati^{2,*,#}, PhDCEngCPhysCSci

¹Neuroimmunology and Neuromuscular Diseases unit, ² Scientific department and ⁴ Neuroradiology unit, Fondazione IRCCS Istituto Neurologico “Carlo Besta”, Milano, Italy;³ Brighton and Sussex Medical School, Brighton, United Kingdom;

These two authors contributed equally to study design and supervision.

Running title: Quantitative magnetization transfer imaging in cuprizone-treated mice

Abstract

The pool size ratio measured by quantitative magnetization transfer MRI (qMT-MRI) is hypothesized to closely reflect myelin density, but their relationship has so far been confirmed mostly in ex vivo conditions. We investigate the correspondence between this parameter measured in vivo at 7.0 Tesla, with black gold II staining for myelin fibres, and with myelin basic protein and beta-tubulin immunofluorescence in a hybrid longitudinal study of C57BL/6 and SJL/J mice treated with cuprizone, a neurotoxicant causing selective myelin loss followed by spontaneous re-myelination upon treatment suspension. Our results confirm that pool size ratio measurements correlate with myelin content, with the correlation coefficient depending on strain and staining method, and demonstrate the in vivo applicability of this MRI technique to experimental mouse models of multiple sclerosis.

Keywords

Quantitative magnetization transfer imaging (qMT); macromolecular pool size ratio (F); multiple sclerosis; animal model; cuprizone; demyelination; remyelination.

Abbreviations

BGII: Black Gold II

F : Macromolecular pool size ratio

CC: Corpus Callosum

Ctx: Cortex

DAPI: Diamidinophenylindole-dihydrochloride

FLASH: Fast Low-Angle SHot

FoV: Field of View

k_f : Forward exchange rate

MBP: Myelin Basic Protein

MFI: Mean Fluorescent Intensity

MRI: Magnetic Resonance Imaging

MT: Magnetization Transfer

NEX: Number of Excitations

PBS: Phosphate Buffer Solution

PFA: Paraformaldehyde

qMT: Quantitative Magnetization Transfer

RARE: Rapid Acquisition with Relaxation Enhancement

ROI: Region of interest

T_{1obs} : Longitudinal relaxation time

T_{2B} : Transverse relaxation time of the macromolecular pool

TE: Echo time

TR: Repetition time

Introduction

A significant challenge to the development of multiple sclerosis therapeutics is the difficulty in assessing myelin status non-invasively. While volumetric measurement of white matter hyper-intensities and diffusion-weighted imaging play an important role in the study of the disease, neither provides a specific direct measure of myelin integrity since these techniques are affected by secondary aspects of the disease such as inflammation and oedema[1].

In contrast, magnetization transfer (MT) imaging has been shown to provide a means of assessing myelin status. This technique employs a two-pool model for the protons in tissue, consisting of a mobile “free water” pool and a semisolid “macromolecular” pool. The protons in the free water pool are directly observable in MRI and are responsible for the signal observed in conventional MR images. On the other hand, the macromolecular pool, which is composed of biologically interesting components such as myelin, is not normally directly observed. However, the two pools are magnetically coupled and it is possible to deduce properties of the macromolecular pool by studying the transfer of magnetization between the pools. This is practically achieved by applying a spectrally-selective pulse to the protons in the macromolecular pool only and observing the reduction in the free water signal as a consequence of magnetization transfer [2]. An approximate measure of myelin density can be obtained by simply calculating the relative water signal attenuation (referred to as magnetization transfer ratio, MTR), associated with bound proton pool saturation. Quantitative MT (qMT) yields a quantitative determination

of the bound proton fraction referred to as f , or equivalently of the macromolecular pool size ratio F , through the application of detailed two- or multi-pool exchange models, which relate f and F to signal attenuation as a function of saturation pulse frequency offset and intensity, as well as T_1 relaxation time [3,4].

An alternative approach consists of fitting multiple components to the T_2 -decay curves; this technique allows closer representation of myelin status compared to acquiring a single image at a given echo time as commonly done in clinical practice. It remains a matter of active debate whether f (and F) or T_2 -derived parameters provide the most accurate measure of myelin status; evidence available to date suggests that the two techniques measure overlapping microstructural features but each have a different sensitivity to inflammation and intact myelin vs. myelin debris, by virtue of the different physical mechanisms involved [5-7]. It should also be noted that, while the apparent diffusion coefficient as determined through mono-exponential fitting is highly sensitive to inflammation and oedema, recent data demonstrate closer correlation between myelin status confirmed histologically and more advanced parameters of non-Gaussian diffusion, such as diffusional kurtosis [8].

Parametric maps obtained using magnetization transfer imaging, referred to as from qMT-MRI, readily identify damage to normal-appearing white matter in multiple sclerosis patients, differentiating phenotypes and closely correlating with the severity of clinical symptoms [7,9]. In recent years, a multitude of studies have investigated the correspondence between myelin status determined histologically, using conventional staining, immuno-staining and

electro-microscopy, and the imaging parameters MTR or f (and, equivalently, F). A strong correspondence has been repeatedly reported for excised tissue from multiple sclerosis patients, as well as mice and rats treated with the copper chelator cuprizone (bis(cyclohexanone) oxaldihydrazone) or with experimentally induced encephalomyelitis (see, for example [10-15]). However, to date comparatively fewer studies have investigated the correspondence between in vivo measures of MTR and f (and F) and myelin status as measured by histology.

It has been demonstrated that, compared to wild-type controls, F is substantially reduced in the white matter of shiverer mice, that have nearly no myelin but have otherwise normal brain cytoarchitecture [16]. Furthermore, axonal loss following retinal ischemia does not lead to reduced values of F in shiverer mice, thus confirming that the sensitivity of this parameter is primarily to myelin and not axonal integrity per se [17]. However, such results were obtained for ex vivo scans, following perfusion and complete tissue fixation, and therefore may not accurately represent in vivo imaging conditions.

Addressing this limitation, we investigated the correspondence between F , measured through in vivo qMT-MRI, and ex vivo histological markers of myelin density and axonal integrity, in two mice strains treated with cuprizone. This experimental toxicant, when administered for brief periods, induces spontaneously reversible demyelination, with limited neural loss and mainly local inflammation and astrogliosis, with more constrained activation of peripheral immune cells compared to experimental autoimmune

encephalomyelitis (EAE) and lipopolysaccharides (LPS) inoculum [13,18,19].

Materials and methods

Animals and treatment

All procedures complied with the 86/609 EEC directive and were part of a project approved by the Italian ministry of health (ref. 31/2011-B, PI: FB). Six-week old C57BL/6 male (n=39) and SJL/J female (n=39) mice were obtained from Charles River (Calco, Italy) and housed ($21\pm 2^{\circ}\text{C}$, humidity $40\pm 2\%$, 12/12-h light/dark cycle) in the accredited animal facility of the Carlo Besta Neurological Institute with food and water provided ad libitum. After 1 acclimation week (baseline), 4 mice were euthanized, 22 were fed cuprizone (0.2% w/w) for 5 (C57BL/6) or 7 weeks (SJL/J) to obtain robust demyelination, and the remaining 13 received regular chow diet. For each strain, after 5/7 weeks, 6 untreated and 6 treated mice were submitted to histology, 8 treated mice returned to normal diet and 8 continued receiving cuprizone until the end of experiment (10 wks). The experimental design was optimized to allow multimodal investigation of demyelination as well as re-myelination following treatment suspension, and is depicted in Supplementary Figure 1; it should be noted that not all mice underwent qMT-MRI, and imaging data were only available for 15 mice (C57BL/6) or 4 mice (SJL/J, the difference being related to the fact that for the present report it was decided to perform planned comparison that did not involve this time-point) at 0 wks, for 15 mice at 5/7 wks and for 11 mice at 10 wks. The experiment was conducted in parallel with C57BL/6 and SJL/J mice, strains frequently employed in multiple sclerosis research, which are characterized by different

sensitivity to cuprizone intoxication, as reflected in the different duration of administration at the intermediate time-point (5 or 7 wks) [13,18,19]. For MRI, mice were anesthetized with 1-2% isoflurane, and positioned on a water-heated bed fitted with a nose-cone for gas delivery and a three-point head fixation system (tooth-bar and ear-plugs). During scanning, the breathing rate and temperature were monitored, respectively with a pneumatic sensor and rectal thermistor, for the purpose of regulating aesthetic concentration and bed heating. For histology, mice were euthanized through deep anesthesia with i.p. chloralium hydrate 4% (Sigma-Aldrich) followed by intracardiac perfusion with PBS (EuroClone, Milan, Italy) and 4% paraformaldehyde (PFA, Merck, Darmstadt, Germany).

qMT-MRI

qMT-MRI was performed at baseline, intermediate time-point (5/7 wks) and experiment end (10 wks), using a Biospec 70/30 USR system (BrukerBioSpin GmbH, Karlsruhe, Germany), having field strength 7.0 Tesla, bore diameter 30 cm, and equipped with a volume linear coil for transmission and a quadrature mouse brain surface coil for reception. Two sections were studied (approximately +0.75 and -1.35 mm to bregma; Supplementary Figure 2), perpendicular to brain base and positioned on the corpus callosum genu and splenium, areas of most marked cuprizone-induced myelin loss [13,18,19]. Accurate longitudinal repositioning was attained by verification of slice location on high-resolution coronal, sagittal and axial anatomical series upon agreement by two operators. MT-weighted images were acquired

using a FLASH sequence having TE=3.3 ms, TR=45.4 ms, excitation angle $\alpha=15.7^\circ$ with Hermitian pulse, NEX=8, slice thickness=600 μm , FoV=19.2x19.2 mm and matrix=192x192. The Gaussian-shaped MT pulses had a duration of 35 ms, 1% truncation, bandwidth 78 Hz and bandwidth factor 2740 Hz ms and excitation angles 1000 and 4000 deg, corresponding to continuous-wave pulse equivalent approximations of 574.3 rad/s and 2297 rad/s respectively, at offset frequencies of 1, 1.36, 1.86, 2.53, 3.44, 4.7, 6.39, 8.71, 11.87, 16.16, 22.02 and 30 kHz [20]. No MT unweighted images were entered in the fitting. T_1 -mapping images were acquired in the same image space as the MT data using a RARE sequence having RARE factor 8, both pulses Hermitian, TE=9.2 ms (equal to effective TE), NEX=1, and TR=600, 800, 1000, 1500, 2200, 3000, 6000 and 8000 ms. For both MT and T_1 -weighted imaging, the two slices were acquired separately.

The effective duration of each MT-weighted FLASH sequence was approximately 52 s, that of the T_1 -weighted RARE sequence was approximately 7 minutes; overall scan time, including pilot sequences, positioning, repeated series was approximately 2 hours per mouse. Following completion of the acquisitions, it was discovered that the T_1 -weighted RARE series were affected by intermittent ghosting artefacts. To reduce the impact of these artefacts on the final parametric maps, all images (corresponding to all mice, slices and TRs) were manually inspected by an experienced operator blinded to treatment group in randomized order, and those where severe artefacts were present were rejected; in total, approximately 9% and 14% of data points were rejected for the rostral and caudal slices respectively.

The model equation used for the qMT was that proposed by Ramani et al. [20], although in contrast with the original paper, the macromolecular pool size ratio F (with $F = f/(1-f)$, wherein the numerical values of F and f are very close given that typical measurements are ~ 0.1) was used and the whole equation was rewritten using F and the forward exchange rate k_f . This is consistent with a number of recent papers from our group and more details can be found in [3,4]. MT parameters were estimated on a voxel-by-voxel basis by fitting this model equation to the data using the Levenberg–Marquardt method. The absorption line-shape of the macromolecular pool was modelled with a super-Lorentzian, whose values were previously computed using a look-up table. T_1 maps were obtained by fitting the spin-echo signal equation $y = M \cdot (1 - \exp(-TR/T_1))$ to the RARE images on a voxel-by-voxel basis, again using the Levenberg–Marquardt method [3].

For each voxel in the parametric maps, the mean and standard deviation in the 5 x 5 neighbourhood were calculated. Voxels having values farther away from the mean than 3 standard deviations were considered outliers and replaced with the median, calculated over the 3 x 3 neighbourhood. This operation enabled removal of fitting outliers without unduly sacrificing spatial content.

Histology and immunofluorescence

Brains of PFA-perfused animals were post-fixed overnight in PFA (4°C), cryo-protected (30% sucrose in PBS, 4°C for 24h; Carlo Erba, Milan, Italy), frozen and transversally sectioned corresponding to

the MRI sections, using a Leica CM1950 cryostat. Histological myelin staining was performed on 20 μm slices using Black Gold II (BGII, Histo-Chem Inc. Jefferson, Arkansas, USA) and stained slices were captured with Aperio digital scanner equipped with 20x objective (ScanScope, Aperio Technologies, CA, USA). Double immunofluorescence was performed on 10 μm slices using rabbit polyclonal anti-MBP (Millipore #AB980, Billerica, Massachusetts, USA) and mouse monoclonal anti- β -tubulin (Covance #TUJ1, Princeton, New Jersey, USA) primary antibodies, followed by Alexa Fluor 488- and 594- conjugated goat anti-rabbit and anti-mouse (Molecular Probes-Invitrogen, Carlsbad, California, USA) secondary antibodies, respectively. Anti- β -tubulin staining fragmentation was evaluated as previously described to provide an index of neural loss [21].

Nuclei were visualized by 4',6-diamidino phenylindole-dihydrochloride staining (DAPI; Chemicon, Temecula, California, USA). Images were captured using a confocal laser-scanning microscope (D-Eclipse C1, Nikon, Tokyo, Japan) equipped with 40x (NA:1.30) oil objective, and acquisition settings were maintained constant for all samples.

Data analysis

The F for qMT-MRI and pixel intensity ratio to background for histology and immunofluorescence were measured using manually-drawn regions of interest (ROIs) positioned over the medial, intermediate and lateral parts of the corpus callosum (CC;

Supplementary Figure 2). The ROIs were drawn by experienced operators blinded to the experimental groups, separately for qMT-MRI and histological images, using the ImageJ software [22]; to accelerate analyses and reduce the probability of committing mistakes, the measurements were extracted using the ROIs through a script developed in-house using the MatLab language. Owing to the comparatively poor spatial resolution of qMT-MRI, for this modality ROI drawing was performed twice to confirm inter-rater agreement, and prior to ROI drawing, parametric maps of F were spatially interpolated by a factor of 2.

The ROI drawing procedure involved the use of a “template” for each of the two sections, which consisted of a square cropping ROI corresponding to the brain outline, containing CC ROIs pre-positioned at representative locations that had been previously determined considering all scans. For each measurement, the operators manually rotated the slice if necessary to align the interhemispheric plane vertically, translated the cropping ROI to match the brain outline as closely as possible, then adjusted the CC ROIs to accurately correspond to its outline as observed on the F maps. The ROI area was fixed to $0.04 / 0.08 \text{ mm}^2$ for medial (rostral / caudal), $0.03 / 0.09 \text{ mm}^2$ for intermediate and $0.03 / 0.06 \text{ mm}^2$ for lateral CC. The operators were instructed to disregard the relative intensity of the CC on the F maps, and concentrate instead on identifying its outline, adjusting the visualization window as necessary to allow for the intensity changes observed as a consequence of demyelination. Usage of 6 small ROIs was preferred to delineation of the whole CC as a single ROI, as positioning circular ROIs is much less problematic than deciding on

the outline of the whole CC, particularly when its borders are blurred due to demyelination, and thus promoted reproducibility. Differently from the MRI images, in histological/immunofluorescence sections with specific myelin stainings, the corpus callosum is easily recognizable and specific ROIs were drawn according to the image field for each section and normalized to area size.

For all modalities, planned comparisons were performed as follows: i) between untreated and treated mice at the 5/7 weeks time-point, ii) between treated mice (5/7 wks) and mice with continued treatment (10 wks), and iii) between treated mice (5/7 wks) and mice with suspended treatment (10 wks). These planned comparisons enabled assessing the primary effect of treatment after a typical time interval (5/7 wks), as well as the aggravation associated with continuation and the spontaneous re-myelination following suspension [13,18,19]. To minimize the probability of type I error, we adopted a conservative approach: we did not perform any other comparisons, and combined all 6 ROIs (and 2 operators for qMT-MRI) into a single median value for each mouse. All comparisons were performed by means of non-parametric two-sided rank sum tests, equivalent to Mann-Whitney U-tests. Relative change was also calculated as the difference between the medians divided by the median of untreated mice at 5/7 wks (for comparison i) or the median of treated mice at 5/7 wks (for comparisons ii and iii).

The focus of the study has been on the pool size ratio F , since we hypothesized this parameter is likely to show direct correlation to myelin status. However, as the qMT model also yields complementary measures, namely the forward exchange rate k_f and transverse

relaxation time of the macromolecular pool T_{2B} , a statistical comparison of these measures was additionally performed to provide a more complete analysis of the experimental data. To enable validation of the measurements, the observed longitudinal relaxation time T_{1obs} was also analyzed. Furthermore, a magnetization transfer ratio (MTR) map was determined by taking the ratio of free water signal intensity at 1.86 kHz offset, 1000 deg flip angle, (representing a moderate intensity of MT weighting, comparable to those used in published MTR studies [3,4]) and the least MT-weighted images (acquired with MT flip angle of 1000 deg and offset frequency of 30 kHz, which were effectively nearly-unweighted).

Separate Spearman rank-order regressions were performed to explore the correlation between F (dependent variable) and the staining intensity with BGII, MBP and β -tubulin (independent variables). In this analysis, all 6 ROIs (and 2 operators for qMT-MRI) were combined with the median for each mouse, and data from all time-points, including those not considered for planned comparisons described above, were entered. A non-parametric approach was chosen to mitigate the effect of potential outliers given the small sample size, as well as to avoid assumptions about linearity of the relationship between F and the histological parameters.

An equivalent analysis was used to determine the agreement of F measurements over the 2 operators for qMT-MRI, which was near-perfect: $\rho=0.93$ and $\rho=0.98$ for C57BL/6 and SJL/J mice respectively.

Results

qMT-MRI

Longitudinal analysis of the CC in cuprizone-treated mice revealed a reduction in F values, suggesting loss of myelin compared to untreated age-matched controls (Figure 1a, d and Figure 2a, d). Myelin derangement, detected at 5/7 weeks of treatment (0.050 ± 0.005 vs. 0.065 ± 0.004 , $p=0.001$, $\Delta=-22.7\%$, and 0.045 ± 0.007 vs. 0.056 ± 0.005 , $p=0.003$, $\Delta=-19.5\%$ for C57BL/6 and SJL/J mice respectively, compared to untreated controls), was aggravated after treatment continuation, consistently resulting in a further F reduction (0.044 ± 0.002 vs. 0.049 ± 0.005 , $p=0.038$, $\Delta=-9.6\%$, and 0.039 ± 0.004 vs. 0.046 ± 0.004 , $p=0.019$, $\Delta=-16.6\%$ for C57BL/6 and SJL/J mice respectively, compared to 5/7 wks treated mice). Treatment suspension resulted in partial recovery of F levels in C57BL/6 mice (Figure 1a, d; 0.056 ± 0.003 vs. 0.049 ± 0.005 , $p=0.019$, $\Delta=14.0\%$, compared to 5 wks treated mice), but not in SJL/J mice (Figure 2a, d). To avoid inserting the same animal twice in these unpaired tests, for both comparisons on treatment continuation and suspension we excluded from the 5/7 weeks sample those animals included at 10 weeks (4 in each case).

Values of k_f followed a similar trend but the changes were more constrained: for C57BL/6 mice (Suppl. Figure 3a) demyelination effects were detected at 5 weeks ($p=0.001$, $\Delta=-14.3\%$) and upon treatment continuation ($p=0.01$, $\Delta=-8.6\%$). However, remyelination after treatment suspension was not significant; for SJL/J mice (Suppl.

Figure 4a), only demyelination at 7 weeks reached significance ($p=0.001$, $\Delta=-19.4\%$).

Changes in T_{2B} showed a different pattern less consistent with that of F , with only an effect of treatment continuation ($p=0.019$, $\Delta=-4.7\%$) for C57BL/6 mice (Suppl. Figure 3b), and an initial decrease at 7 weeks ($p=0.001$, $\Delta=-6.4\%$) and recovery after treatment suspension ($p=0.019$, $\Delta=2.1\%$) for SJL/J mice (Suppl. Figure 4b).

The MTR ratio revealed changes in line with F , but was less sensitive: demyelination following initial treatment at 5 weeks ($p=0.001$, $\Delta=-8.8\%$) and 7 weeks ($p=0.001$, $\Delta=-11.7\%$) respectively for C57BL/6 and SJL/J mice (Suppl. Figure 3c, 4c) was detected, but no other effects reached statistical significance.

The T_{1obs} followed the converse trend, increasing after 5 weeks of treatment ($p=0.001$, $\Delta=7.3\%$) and further upon treatment continuation ($p=0.01$, $\Delta=5.2\%$), and decreasing following treatment suspension ($p=0.038$, $\Delta=-4.6\%$) for C57BL/6 mice (Suppl. Figure 3d); no significant differences were found for SJL/J mice (Suppl. Figure 4d).

Histology

BGII histology demonstrated demyelination in both strains (Figure 1b, e and Figure 2b, e; $p=0.002$, $\Delta=-21.6\%$ and $p=0.002$, $\Delta=-13.2\%$ for C57BL/6 and SJL/J mice respectively, compared to untreated controls), and remyelination in C57BL/6 mice only (Figure 1b, e; $p=0.001$, $\Delta=24.8\%$, compared to 5wks treated mice). No effect of treatment continuation was detected by this staining.

MBP immunofluorescence confirmed cuprizone-induced demyelination in both strains (Figure 1c, f and Figure 2c, f; $p=0.002$, $\Delta=-37.4\%$ and $p=0.002$, $\Delta=-39.3\%$ for C57BL/6 and SJL/J mice respectively, compared to untreated controls) and its aggravation with continued treatment (Figure 1c, f and Figure 2c, f; $p=0.043$, $\Delta=-9.4\%$ and $p=0.026$, $\Delta=-3.5\%$ for C57BL/6 and SJL/J mice respectively, compared to 5/7 wks treated mice). It also indicated recovered production of at least one myelin sheath protein even in SJL/J mice (Figure 1c, f and Figure 2c, f; $p=0.001$, $\Delta=87.5\%$ and $p=0.001$, $\Delta=74.7\%$ for C57BL/6 and SJL/J mice respectively, compared to 5/7 wks treated mice).

According to β -tubulin immunofluorescence, C57BL/6 mice exhibited significant neuronal loss following treatment (Figure 1 c, g; $p=0.002$ and $\Delta=-22.9\%$ compared to untreated controls) and significant neuronal recovery following suspension (Figure 1 c, g; $p=0.02$ and $\Delta=22.6\%$, compared to 5 wks treated mice), without aggravation after treatment continuation; no significant effects were detected for SJL/J mice.

Correlation

Rank-order correlations demonstrated significant positive F association with BGII and MBP in C57BL/6 mice (Figure 3a, b; $\rho=0.743$, $p=0.002$ and $\rho=0.589$, $p=0.023$ respectively) and in SJL/J mice (Figure 3a, b; $\rho=0.586$, $p=0.024$ and $\rho=0.536$, $p=0.042$ respectively). Correlation between F and β -tubulin was observed in C57BL/6 mice only (Figure 3c; $\rho=0.668$, $p=0.008$).

Discussion

A defining feature of cuprizone-induced demyelination is that some secondary effects, such as inflammation, oedema or toxicity, are more limited in comparison to other models, such as EAE or LPS inoculum. Contingent to appropriate dosage and administration duration, the effect of cuprizone intoxication is a selective and reversible loss of oligodendrocytes, which has been particularly observed in the corpus callosum so far [13,18,19,23,24]. Our finding of significant pool size ratio reduction during treatment and recovery after suspension, together with significant correlation with histological measurements, therefore corroborates previous *ex vivo* imaging work on shiverer mice demonstrating that this parameter is significantly linked to myelin status [16,17].

In particular, the findings of the present study are in line with a recent *in vivo* investigation at 7.0 Tesla, wherein it was found that the magnetization transfer ratio correlates with progressive myelin loss during cuprizone treatment in the corpus callosum and deep white matter [25]. Our results confirm and extend this investigation in multiple ways. First, we report correspondence with the pool size ratio, which is a quantitative parameter, estimated by fitting a two-pool model of exchange between liquid and semi-solid pool protons over a range of excitation offsets and angles. By contrast, the magnetization transfer ratio is known to be highly affected by the sequence acquisition parameters and by T_1 changes, and as such is more liable to confounding effects of tissue changes unrelated to myelin integrity [2-4,20]. Second, we provide an explicit comparison

between two murine strains commonly used in multiple sclerosis research: SJL/J, which are susceptible to relapsing-remitting EAE and to Theiler's murine virus-induced encephalomyelitis [26] but less responsive to cuprizone intoxication [19], and C57BL/6, which are more widely represented in the cuprizone literature [13,17,18,23].

Another recent study reported quantitative magnetization transfer data from rat brains scanned at 9.4 Tesla. Focal demyelinating lesions were induced by intracerebral administration of LPS, and pool size ratio measurements performed *ex vivo* were found to correlate with luxol fast blue histology more strongly than diffusional measurements [15]. The key difference with respect to their work is that here a protocol suitable for *in vivo* implementation was utilized, having scan time ~2 hours vs. >10 hours. Demonstration of accurate measurement of myelin status *in vivo* is particularly important, as the main motivation for using quantitative magnetization transfer in preclinical studies is the possibility to perform longitudinal measurements, increasing statistical power and reducing the number of animals. It should also be considered that the two models are substantially different: while LPS induce focal lesions, cuprizone causes much more subtle and more diffuse demyelination, more closely resembling the changes observed in normal-appearing tissue in idiopathic disease [13]

While statistically significant correlations between pool size ratio and histological measurements were obtained, it is important to acknowledge that the correspondence was not consistent across all our data and the correlation coefficient depended on the mouse strain and histological staining method. Additionally, due to the small sample

size we only considered rank-order correlation and the exact transfer function, i.e. whether the relationship is linear or not, remains to be confirmed. At present, qMT-MRI cannot replace histology altogether, not only because of the resolution limitation of MRI, but also because pool size ratio measurements are unavoidably affected by factors other than myelin density due to a simplistic model (two-pools are only an approximation) and limitations such as T_1 -weighting effects, inhomogeneous B_1 , error in the attained excitation angles due to non-linearity etc. and measurement noise [3,4,20]. A particular element of concern is the inflammation which is, albeit more constrained in comparison to other models, invariably present in response to cuprizone administration, already at very early time points and as also confirmed in a recent study on diffusional kurtosis imaging [8,27]. We are unable to exclude a confounding effect of inflammatory changes on pool size ratio F readings, and additional investigation is therefore necessary to specifically address the question of whether F is truly less sensitive than T_2 and diffusional measurements to inflammation, using time-points optimized to capture the full dynamic range of inflammatory changes at the beginning and after suspension of cuprizone administration.

We observed significant de-myelination during the initial period of treatment (5 or 7 wks) as well as aggravation for continued treatment at 10 weeks for both strains, but spontaneous re-myelination was evident only for C57BL/6 mice. While histology with black gold II confirmed this pattern, significant re-myelination was detected with myelin basic protein immunofluorescence also for SJL/J. This discrepancy is plausibly related to the inherent differences in

cuprizone-susceptibility of the strains [19], as well as to the mismatched recovery time allowed after treatment suspension before the final experimental time-point. This mismatch was due to choosing a fixed overall maximum duration of administration (10 wks.) while having different timings of treatment continuation/suspension. The choice to have the intermediate time-point at 5 or 7 wks according to the employed strain was derived from known differences in the cuprizone susceptibility of the two strains, and indeed was important in enabling de-myelination to be observed in SJL/L mice. Unfortunately, there was an undesired reduction in the time allowed for re-myelination in this strain [18,19].

The fact that significant alterations in pool size ratio were detected even in SJL/J mice confirms that our implementation of qMT-MRI on an experimental 7.0 Tesla scanner is highly sensitive, given the smaller relative size of the changes expected in this strain [19]. While presence of a significant reduction in β -tubulin levels in C57BL/6 mice may appear unexpected, previous studies have demonstrated that axonal damage and neuronal loss inexorably follow demyelination in the most affected regions when treatment is protracted; this is not interpreted as an indication of direct toxicity, but as an unavoidable consequence of the physiological reliance of neurons on oligodendrocytes [18,28,29]. Importantly, the correlation between pool size ratio and black gold II staining was stronger, and clearly significant also for the SJL/J strain, for which β -tubulin levels were clearly unaffected. In this regard, combined study of the two strains enabled us to firmly conclude that pool size ratio variations are

primarily and selectively driven by myelin degradation, not by neuronal loss.

The pool size ratio F has been shown to have superior sensitivity to cuprizone-induced de-myelination and re-myelination than k_f , T_{2b} , T_{1obs} or MTR. Moreover, F is preferable since it has a specific relevance to myelin concentration. In contrast, MTR is a relatively unspecific parameter, since it is influenced not only by the pool size ratio but also by the exchange rate and liquid pool relaxation time. In spite of this, MTR continues to be widely used in clinical and preclinical studies, thanks to its rapid acquisition time and simple computation. Our observation of greater sensitivity of F to de- and re-myelination is in agreement with two previous studies, where a direct comparison was performed in healthy volunteers and in an EAE model, and diverse F values were observed for structures that had similar MTR values [11,30]. However, it should be noted that the MTR was only calculated for a single, intermediate level of MT weighting, and an exhaustive analysis of the effect of weighing intensity on sensitivity was not conducted; further, there is emerging evidence that the determinants of this contrast may be different at high field strength [31]. The biological interpretation of k_f and T_{2b} is less clear; there is some evidence that k_f may be related to metabolic changes, and that T_{2b} , which generally shows small and inconsistent changes in pathology, may be influenced by fibre orientation [9,32,33].

In this study, the myelin content was assessed using BGII histology and MBP immunofluorescence. Both techniques are widely used, and sensitive to intact myelin as well as myelin debris; however,

while immunofluorescence is an antibody-based assay, aiming to detect a single protein of the complex myelin multi-lamellar structure, the gold particles of BGII stain many targets (including MBP) in the inner, the compact and the outer layers of myelin structure with the same affinity [34-36]. The authors are not aware of quantitative data on their relative sensitivity to demyelination, and it remains to be determined whether either staining, or imaging measures such as MTR or F , scale linearly with actual myelin content. It should also be noted that, while F is hypothesized to be more directly linked to myelin content than other imaging parameters, its quantification is not totally independent of experimental settings and the macromolecular pool it probes also contains, albeit in substantially lower concentration, other molecules harbouring bound protons [3,4,9,20].

The present investigation has limitations that should be considered. First, we pooled together data from all regions and did not provide any information on regional changes in differing white and gray matter areas. We deliberately adopted this conservative approach both to limit the number of multiple comparisons, given the small sample size and consequently limited statistical power, and to acknowledge the presence of artefacts on some images, as the experimental hypotheses concerned the correspondence between qMT-MRI and histological measurements, and not regional effects, which have already been investigated in detail elsewhere [8,14,23,25,37,38].

Second, values of F are comparatively low, even for untreated mice, in comparison to in vivo studies on humans as well as ex vivo data in mice: typical values are ~ 0.1 , whereas here controls had values

~ 0.05 [7,9,16,17]. The most probable explanation for this discrepancy is the fact that, given the high field strength, residual T_1 -weighting was present in the MT-sensitized images. This effect could be mitigated with longer repetition times and smaller flip angles, but this would have protracted the imaging sessions prohibitively or reduced signal level unacceptably. Ramani's model does not take into account residual T_1 -weighting and this is the likely reason for the systematic under-estimation of the bound proton pool in this work. It was previously shown that F is progressively underestimated when the amount of T_1 -weighting increases [4]. T_1 saturation is not cancelled out by equal weighting of all scans because, while the macromolecular pool is saturated (fully or partially), the apparent longitudinal relaxation time of the system is shorter than that measured in the absence of MT saturation [39]. As the 24 scans used to estimate the model parameters are acquired with variable amounts of MT-weighting, they will be differently affected by T_1 saturation. This effect has been clearly shown by numerical simulations in [4], where F values measured in the healthy human brain were found to decrease when using a flip angle of 15° instead of 5° , with otherwise identical parameters, in a FLASH sequence with $TR=28\text{ms}$ at $1.5T$.

Here, Ramani's model was preferred over Sled and Pike's models because of its more favourable sensitivity to signal-to-noise ratio, and of its generally simpler and more stable fitting process [4,20]. Since the purpose of the study was to conduct an internal comparison between data acquired with the same protocol, and particularly to perform longitudinal and correlational analysis, the

more stable model was preferred, at the expense of introducing systematic bias in the measurements.

This study was performed at 7.0 Tesla, and, while the increased signal-to-noise ratio improves measurement accuracy, qMT-MRI poses some additional challenges at high field strengths: the T_1 relaxation times are longer, giving rise to greater errors due to residual T_1 weighting compared to lower field strengths; the power levels required to attain a given flip angle are increased, resulting in practical limitations for in vivo applications, and due to the shorter wavelength B_1 field inhomogeneities can introduce greater error than at lower frequencies [3]. Indeed, it is well known that it is difficult to model the behaviour of magnetization at low off-resonance frequencies in pulsed MT experiments. While a minimum offset of 1kHz has been determined empirically at 1.5 Tesla [40], this is likely to vary with field strength and MT pulse amplitude and bandwidth. The optimal distribution of offset frequencies at 7.0 Tesla will thus need to be comprehensively investigated in future studies. In this study, the B_1 field was not mapped, leaving the possibility of spatial gradients that may influence the qMT measurements.

Our study has potential practical implications at two levels. First, it reaffirms the sensitivity and biological specificity of pool size ratio measurements, suggesting that qMT-MRI should be used as a key outcome measure in trials of multiple sclerosis therapeutics. Second, it demonstrates that qMT-MRI can be efficiently implemented in vivo on a small-animal scanner and that its sensitivity to myelin changes is comparable to histological measures, prompting

its consideration as a primary investigational tool in preclinical research on murine models of multiple sclerosis.

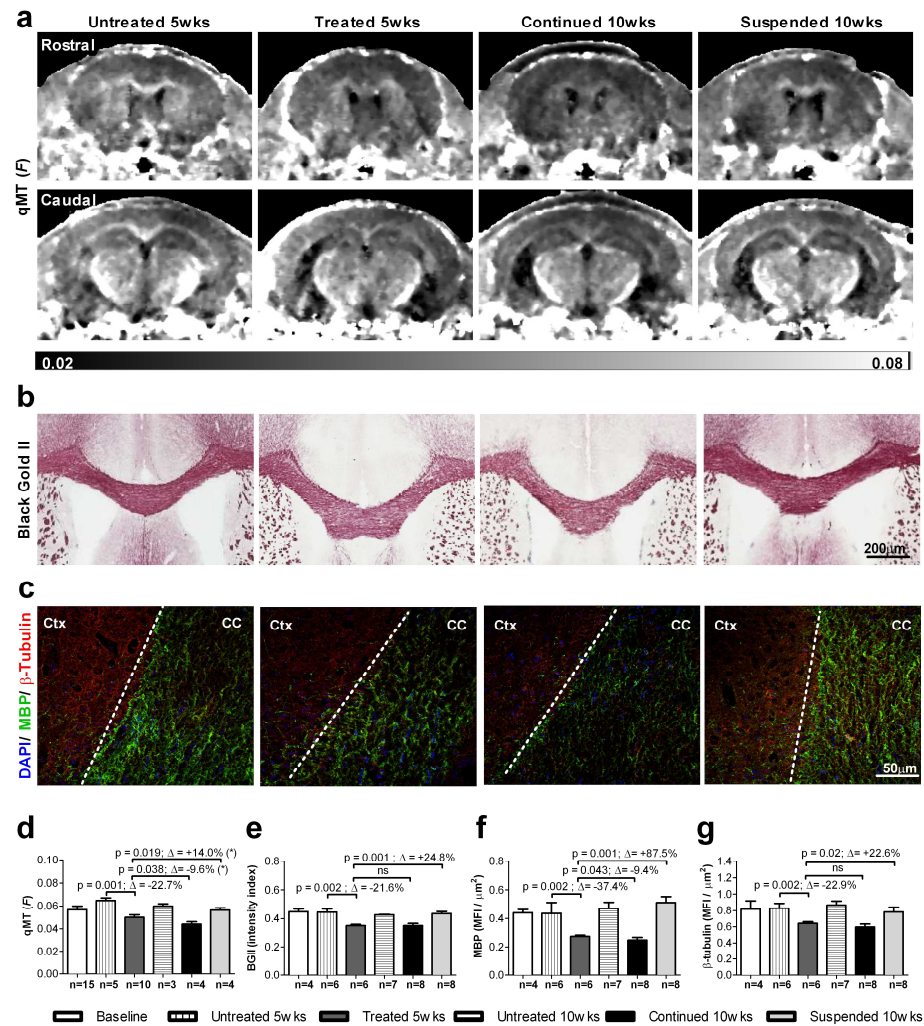


Figure 1.

Figure 1. Representative sections from brains of C57BL/6 male mice prior to treatment, at 5 weeks, at 10 weeks after treatment continuation and at 10 weeks after treatment suspension: (a) pool size ratio (F) parametric maps, (b) black gold II (BGII) histology, (c) myelin basic protein (MBP, green) and β -tubulin (red) immunofluorescence (MFI, mean fluorescent intensity) for rostral and caudal areas of corpus callosum (CC) juxtaposed to the cortex (Ctx). Bottom row, bar charts showing median $\pm$ interquartile range for F (d), BGII (e), MBP (f) and β -tubulin (g). Significance values represent planned comparisons performed using Mann-Whitney U-tests. For F (d), to avoid inserting the same animal twice in these unpaired tests, for both comparisons between 5 and 10 weeks indicated by (*) we excluded from the 5 weeks sample those animals included at 10 weeks (4 in each case). It should be noted that not all mice underwent qMT-MRI.

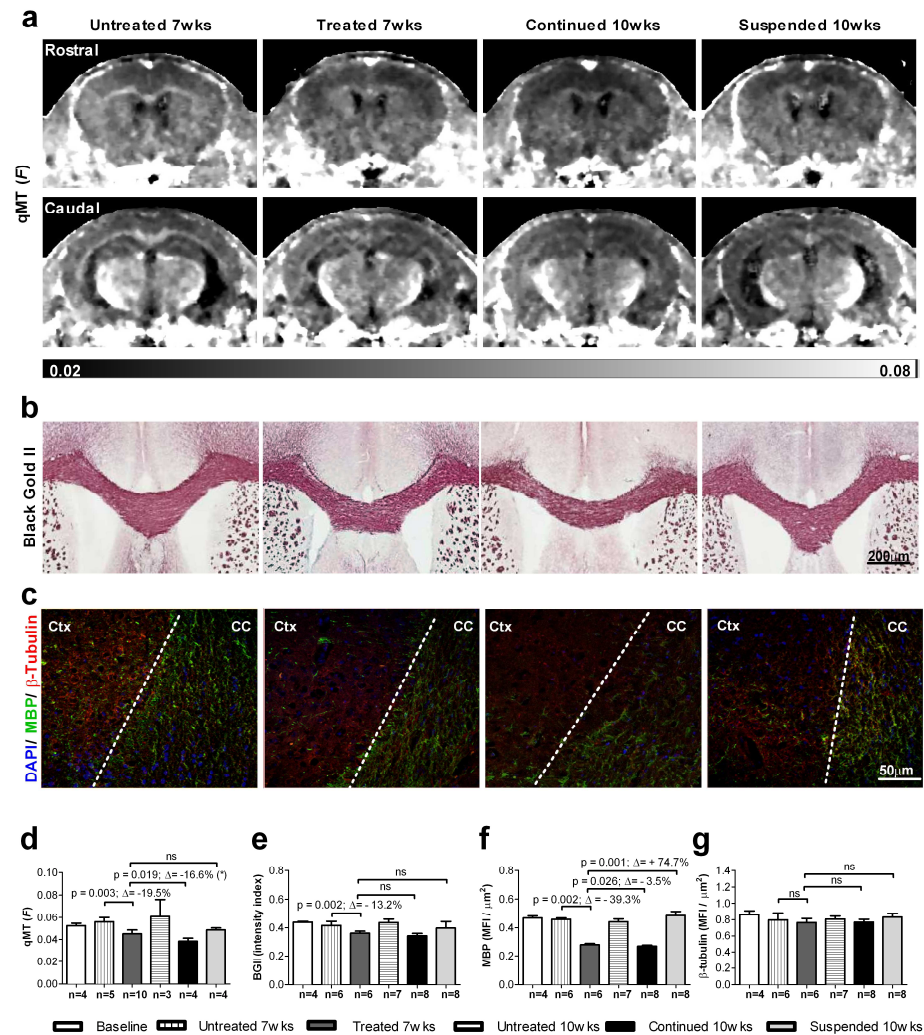


Figure 2.

Figure 2. Representative sections from brains of SJL/J female mice prior to treatment, at 7 weeks, at 10 weeks after treatment continuation and at 10 weeks after treatment suspension: (a) pool size ratio (F) parametric maps, (b) black gold II (BGII) histology, (c) myelin basic protein (MBP, green) and β -tubulin (red) immunofluorescence (MFI, mean fluorescent intensity) for rostral and caudal areas of corpus callosum (CC) juxtaposed to the cortex (Ctx). Bottom row, bar charts showing median $\pm$ interquartile range for F (d), BGII (e), MBP (f) and β -tubulin (g). Significance values represent planned comparisons performed using Mann-Whitney U-tests. For F (d), to avoid inserting the same animal twice in these unpaired tests, for both comparisons between 7 and 10 weeks indicated by (*) we excluded from the 7 weeks sample those animals included at 10 weeks (4 in each case). It should be noted that not all mice underwent qMT-MRI.

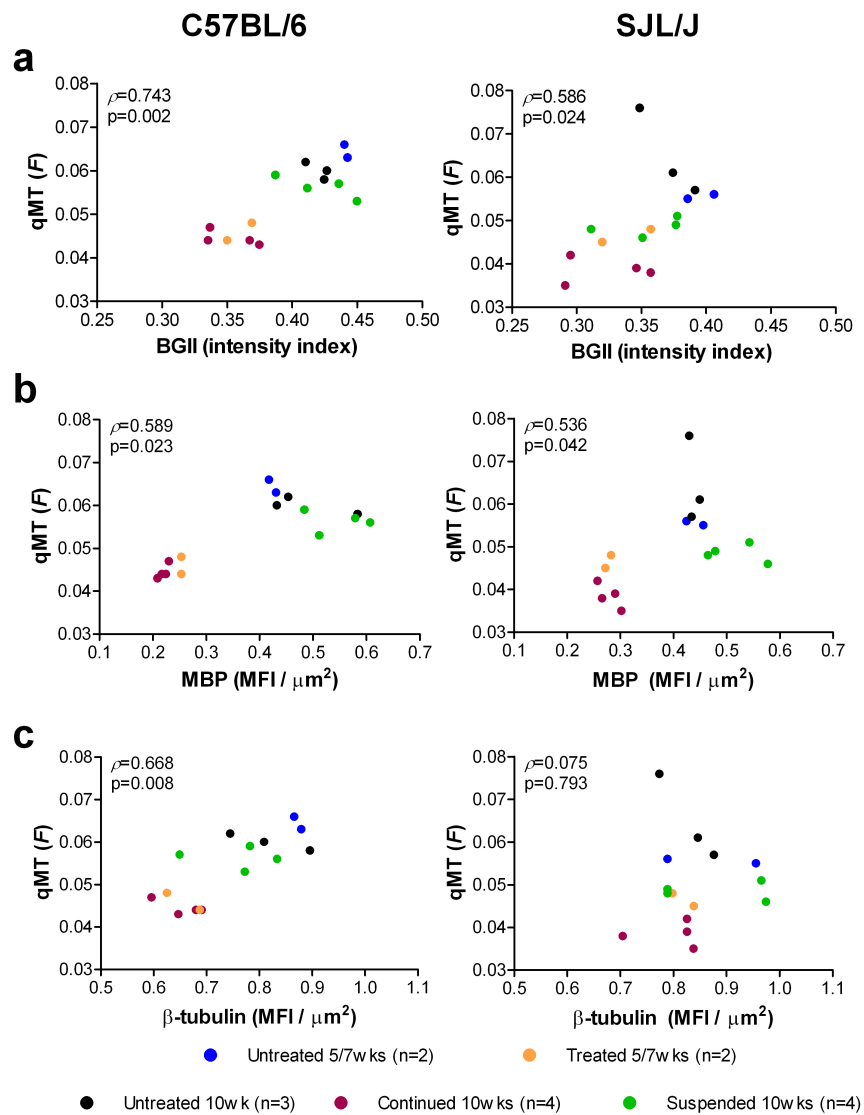
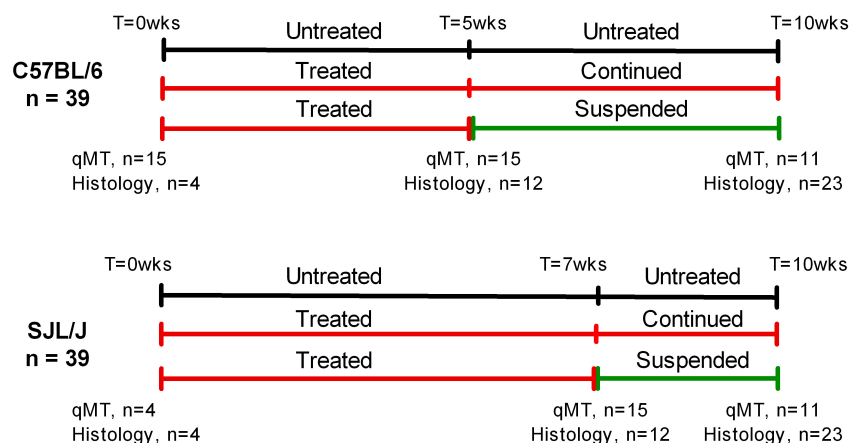
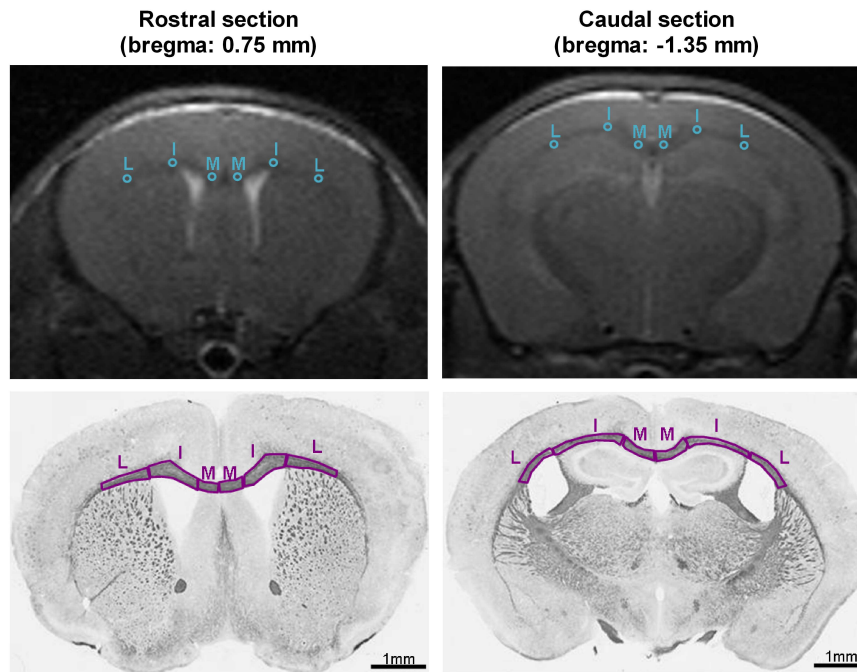


Figure 3.

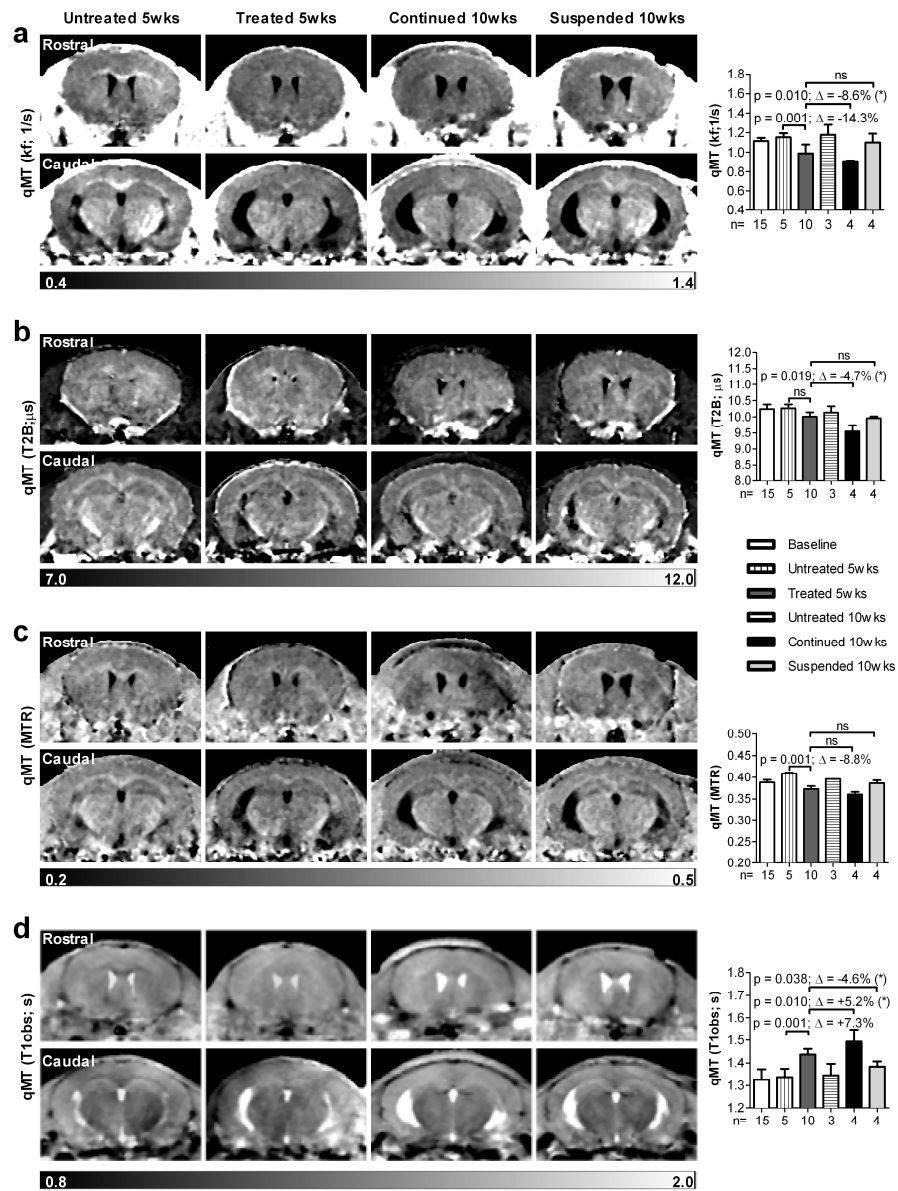
Figure 3. Scatter plots for correlation between pool size ratio (F , dependent variable) and the following independent variables: (a) black gold II (BGII) staining, (b) myelin basic protein immunofluorescence and β -tubulin immunofluorescence (c; MFI, mean fluorescent intensity), shown separately for C57BL/6 male (left) and SJL/J female (right) mice. Each point represents the median over all ROIs (and raters, for F) for each mouse; ρ and p were derived from Spearman rank-order correlation. Only mice who underwent both qMT-MRI and histology at a given time point could be included; thus the numerosity at each time-point is different in comparison that indicated in Figure 1 and 2 for qMT-MRI and histology separately.



Supplementary Figure 1. Study design time-line: normal diet (black line; untreated), cuprizone administration (red line; treated), and cuprizone withdrawal (green line; recovery). This hybrid cross-sectional/longitudinal scheme allowed us to explore de- and remyelination longitudinally with qMT-MRI and at the same time establish correspondence with histological measures. Total number of mice scanned and submitted to histological analysis shown for each time-point (T=0; T=5/7; T=10), for both C57BL/6 (upper panel; n=39) and SJL/J (lower panel; n=39) mice. It should be noted that not all mice underwent qMT-MRI.

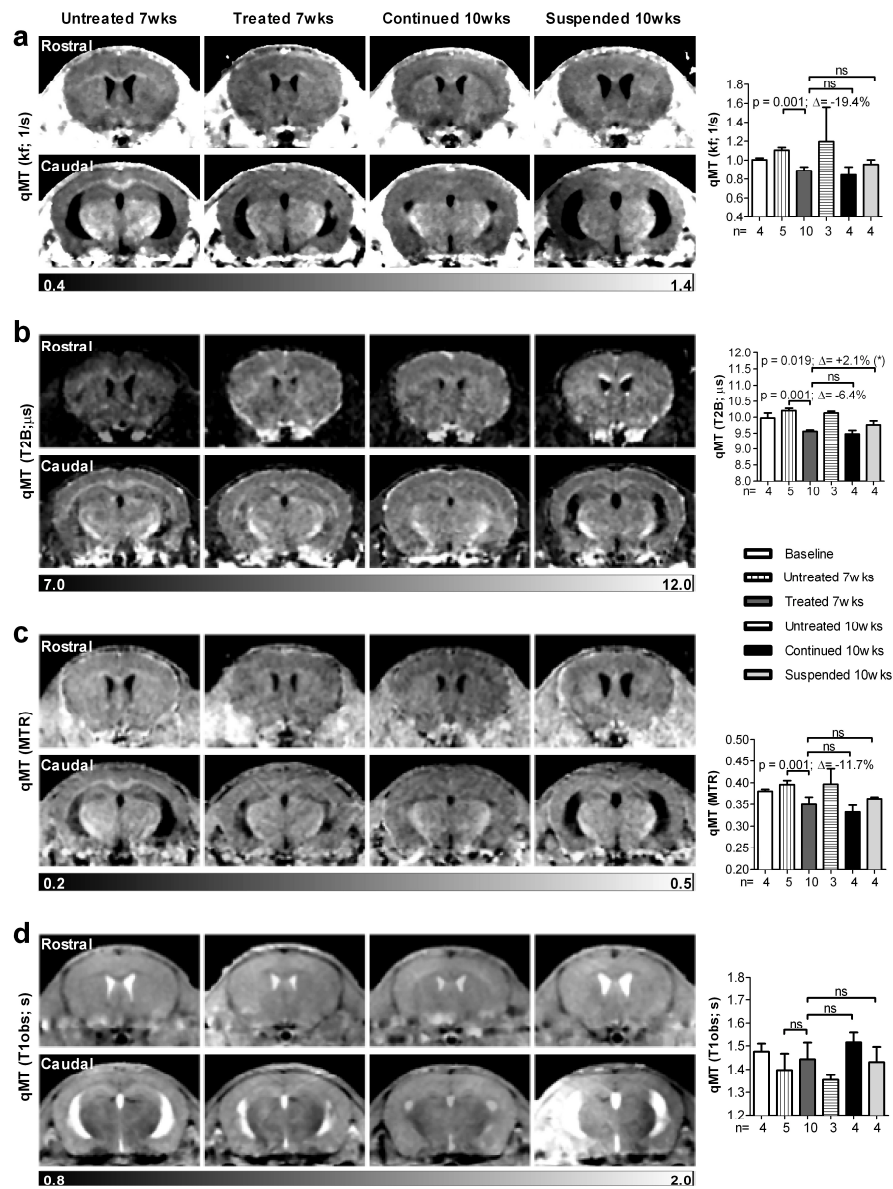


Supplementary Figure 2. Location of regions-of-interest (ROIs) for qMT-MRI (top panels) and histological/immunofluorescence (bottom panel) measurements. Letters “M”, “I” and “L” denote medial, intermediate and lateral locations, which were then averaged together for statistical analysis. The rostral section (left) was positioned ~0.75 mm to bregma, the caudal section (right) was positioned ~-1.35 mm to bregma.



Supplementary Figure 3.

Supplementary Figure 3. Additional representative sections from brains of C57BL/6 male mice prior to treatment, at 5 weeks, at 10 weeks after treatment continuation and at 10 weeks after treatment suspension, and corresponding bar charts showing median $\pm$ interquartile range, for the forward exchange rate k_f (a), transverse relaxation time of the macromolecular pool T_{2B} (b), magnetization transfer ratio (MTR; c) determined at 1.86 kHz offset, 1000 deg flip angle), and observed longitudinal relaxation time T_{1obs} (d). Significance values represent planned comparisons performed using Mann-Whitney U-tests. To avoid inserting the same animal twice in these unpaired tests, for both comparisons between 5 and 10 weeks indicated by (*) we excluded from the 5 weeks sample those animals included at 10 weeks (4 in each case). It should be noted that not all mice underwent qMT-MRI.



Supplementary Figure 4.

Supplementary Figure 4. Additional representative sections from brains of SJL/J female mice prior to treatment, at 7 weeks, at 10 weeks after treatment continuation and at 10 weeks after treatment suspension, and corresponding bar charts showing median $\pm$ interquartile range, for the forward exchange rate k_f (a), transverse relaxation time of the macromolecular pool T_{2B} (b), magnetization transfer ratio (MTR; c) determined at 1.86 kHz offset, 1000 deg flip angle), and observed longitudinal relaxation time T_{1obs} (d). Significance values represent planned comparisons performed using Mann-Whitney U-tests. To avoid inserting the same animal twice in these unpaired tests, for both comparisons between 7 and 10 weeks indicated by (*) we excluded from the 7 weeks sample those animals included at 10 weeks (4 in each case). It should be noted that not all mice underwent qMT-MRI.

Supplementary Table 1.

C57BL/6			Rostral section					
			M	I	L	M	I	L
<i>F</i>	Baseline	median	0.064	0.060	0.054	0.065	0.062	0.059
		IQR	0.011	0.010	0.009	0.008	0.005	0.012
	Untreated 5wks	median	0.077	0.067	0.057	0.066	0.064	0.062
		IQR	0.014	0.010	0.012	0.011	0.002	0.003
	Treated 5wks	median	0.050	0.055	0.042	0.054	0.058	0.046
		IQR	0.013	0.007	0.006	0.009	0.008	0.004
	Untreated 10wks	median	0.069	0.062	0.051	0.061	0.066	0.067
		IQR	0.010	0.005	0.004	0.003	0.013	0.009
	Treated 10wks	median	0.049	0.045	0.036	0.047	0.043	0.039
		IQR	0.005	0.004	0.005	0.008	0.003	0.004
	Suspended 10wks	median	0.063	0.061	0.051	0.059	0.060	0.053
		IQR	0.006	0.009	0.013	0.012	0.007	0.009
kf; 1/s	Baseline	median	1.222	1.219	1.086	1.186	1.138	1.105
		IQR	0.124	0.106	0.155	0.118	0.126	0.110
	Untreated 5wks	median	1.365	1.174	1.216	1.382	1.183	1.095
		IQR	0.120	0.092	0.053	0.255	0.107	0.097
	Treated 5wks	median	1.093	1.144	0.863	1.055	1.105	0.891
		IQR	0.048	0.095	0.131	0.082	0.152	0.097
	Untreated 10wks	median	1.145	1.175	1.075	1.262	1.437	1.188
		IQR	0.318	0.199	0.037	0.146	0.225	0.103
	Treated 10wks	median	0.912	0.973	0.889	0.984	0.893	0.688
		IQR	0.046	0.034	0.082	0.079	0.018	0.077
	Suspended 10wks	median	1.259	1.248	1.066	1.270	1.146	0.919
		IQR	0.218	0.175	0.082	0.053	0.216	0.066
T2B; μ s	Baseline	median	10.156	10.426	9.606	10.119	10.398	10.057
		IQR	0.315	0.659	0.672	0.496	0.441	0.473
	Untreated 5wks	median	9.875	10.097	9.920	10.249	10.691	9.720
		IQR	0.087	0.495	0.475	0.462	0.856	1.030
	Treated 5wks	median	9.844	10.329	9.634	9.992	10.548	9.800
		IQR	0.453	0.527	0.375	0.643	0.444	0.584
	Untreated 10wks	median	10.135	10.858	9.979	10.019	10.911	9.552
		IQR	0.858	0.664	0.948	0.527	0.257	0.714
	Treated 10wks	median	9.357	9.686	9.446	9.560	9.748	9.159
		IQR	0.383	0.628	0.417	0.677	0.194	0.142
	Suspended 10wks	median	9.441	10.137	9.645	10.175	10.475	9.811
		IQR	0.647	0.477	0.559	0.530	0.575	0.289

MTR	Baseline	median	0.398	0.401	0.359	0.384	0.406	0.395
		IQR	0.042	0.038	0.053	0.037	0.031	0.034
	Untreated 5wks	median	0.429	0.418	0.388	0.401	0.408	0.402
		IQR	0.027	0.008	0.070	0.036	0.030	0.047
	Treated 5wks	median	0.375	0.382	0.358	0.375	0.395	0.353
		IQR	0.013	0.015	0.066	0.033	0.045	0.034
	Untreated 10wks	median	0.355	0.396	0.366	0.394	0.401	0.419
		IQR	0.043	0.018	0.010	0.021	0.029	0.035
	Treated 10wks	median	0.377	0.365	0.340	0.402	0.359	0.343
		IQR	0.016	0.046	0.074	0.016	0.015	0.015
	Suspended 10wks	median	0.387	0.389	0.348	0.397	0.401	0.387
		IQR	0.030	0.022	0.025	0.014	0.010	0.018
T10bs; s	Baseline	median	1.243	1.331	1.357	1.247	1.319	1.357
		IQR	0.083	0.070	0.073	0.063	0.065	0.030
	Untreated 5wks	median	1.247	1.333	1.368	1.252	1.341	1.379
		IQR	0.027	0.076	0.037	0.007	0.047	0.032
	Treated 5wks	median	1.403	1.419	1.541	1.395	1.399	1.465
		IQR	0.060	0.092	0.097	0.066	0.095	0.090
	Untreated 10wks	median	1.329	1.368	1.445	1.312	1.273	1.299
		IQR	0.100	0.045	0.048	0.050	0.079	0.053
	Treated 10wks	median	1.428	1.445	1.500	1.449	1.512	1.682
		IQR	0.045	0.061	0.032	0.119	0.053	0.124
	Suspended 10wks	median	1.324	1.328	1.431	1.356	1.368	1.471
		IQR	0.044	0.111	0.064	0.030	0.104	0.084

C57BL/6			Caudal section					
			M	I	L	M	I	L
F	Baseline	median	0.060	0.049	0.052	0.062	0.052	0.056
		IQR	0.006	0.005	0.007	0.006	0.005	0.006
	Untreated 5wks	median	0.068	0.056	0.056	0.070	0.055	0.061
		IQR	0.003	0.009	0.011	0.004	0.013	0.008
	Treated 5wks	median	0.051	0.047	0.046	0.052	0.049	0.055
		IQR	0.006	0.005	0.005	0.003	0.008	0.012
	Untreated 10wks	median	0.066	0.051	0.049	0.068	0.056	0.063
		IQR	0.008	0.005	0.002	0.002	0.004	0.006
	Treated 10wks	median	0.046	0.041	0.042	0.047	0.044	0.048
		IQR	0.004	0.002	0.003	0.001	0.004	0.002
	Suspended 10wks	median	0.056	0.047	0.054	0.056	0.055	0.056
		IQR	0.002	0.003	0.004	0.004	0.009	0.019

kf; 1/s	Baseline	median	1.203	1.050	1.041	1.172	1.008	0.892
		IQR	0.143	0.105	0.104	0.078	0.102	0.076
	Untreated 5wks	median	1.264	1.038	1.024	1.202	1.007	1.034
		IQR	0.106	0.093	0.099	0.171	0.164	0.034
	Treated 5wks	median	1.105	0.961	0.949	1.019	0.921	0.899
		IQR	0.197	0.063	0.107	0.165	0.098	0.105
	Untreated 10wks	median	1.349	1.169	1.031	1.357	1.057	1.155
		IQR	0.197	0.109	0.132	0.045	0.096	0.051
	Treated 10wks	median	0.994	0.944	0.852	1.015	0.866	0.826
		IQR	0.087	0.075	0.097	0.047	0.042	0.070
	Suspended 10wks	median	1.238	1.057	1.039	1.255	1.007	0.915
		IQR	0.151	0.050	0.185	0.064	0.059	0.078
T2B; μ s	Baseline	median	10.490	10.357	10.142	10.332	10.517	10.213
		IQR	0.371	0.280	0.165	0.302	0.281	0.252
	Untreated 5wks	median	10.276	10.263	10.213	10.451	10.429	10.212
		IQR	0.511	0.172	0.468	0.114	0.225	0.399
	Treated 5wks	median	9.951	9.776	10.109	10.033	10.221	10.206
		IQR	0.396	0.155	0.725	0.305	0.382	0.293
	Untreated 10wks	median	10.158	10.023	9.876	10.173	10.091	10.661
		IQR	0.122	0.302	0.175	0.412	0.052	0.050
	Treated 10wks	median	9.601	9.765	9.794	9.481	10.057	9.749
		IQR	0.120	0.121	0.389	0.313	0.117	0.227
	Suspended 10wks	median	9.981	9.780	10.079	10.024	10.099	10.075
		IQR	0.482	0.243	0.365	0.187	0.190	0.552
MTR	Baseline	median	0.392	0.375	0.376	0.396	0.381	0.395
		IQR	0.021	0.030	0.034	0.023	0.021	0.036
	Untreated 5wks	median	0.424	0.399	0.392	0.424	0.399	0.407
		IQR	0.009	0.012	0.002	0.014	0.020	0.026
	Treated 5wks	median	0.369	0.365	0.360	0.369	0.367	0.370
		IQR	0.016	0.016	0.029	0.012	0.036	0.023
	Untreated 10wks	median	0.408	0.377	0.356	0.425	0.396	0.409
		IQR	0.025	0.008	0.040	0.012	0.012	0.022
	Treated 10wks	median	0.356	0.358	0.353	0.361	0.363	0.366
		IQR	0.011	0.017	0.015	0.027	0.005	0.033
	Suspended 10wks	median	0.383	0.359	0.370	0.397	0.382	0.398
		IQR	0.011	0.021	0.017	0.015	0.013	0.031

T1obs; s	Baseline	median	1.320	1.377	1.365	1.363	1.452	1.459
		IQR	0.079	0.041	0.093	0.073	0.077	0.049
	Untreated 5wks	median	1.306	1.421	1.417	1.332	1.397	1.501
		IQR	0.046	0.025	0.013	0.119	0.069	0.086
	Treated 5wks	median	1.395	1.460	1.433	1.443	1.507	1.528
		IQR	0.049	0.030	0.071	0.096	0.065	0.083
	Untreated 10wks	median	1.323	1.425	1.359	1.371	1.404	1.435
		IQR	0.032	0.049	0.055	0.072	0.055	0.076
	Treated 10wks	median	1.489	1.524	1.528	1.523	1.598	1.568
		IQR	0.090	0.068	0.137	0.017	0.043	0.030
	Suspended 10wks	median	1.349	1.429	1.397	1.408	1.486	1.493
		IQR	0.032	0.048	0.122	0.043	0.101	0.104

SJL/J			Rostral section					
			M	I	L	M	I	L
F	Baseline	median	0.058	0.057	0.043	0.055	0.058	0.050
		IQR	0.029	0.006	0.015	0.004	0.009	0.005
	Untreated 7wks	median	0.062	0.055	0.052	0.057	0.062	0.054
		IQR	0.007	0.003	0.009	0.007	0.007	0.006
	Treated 7wks	median	0.049	0.041	0.037	0.051	0.049	0.043
		IQR	0.004	0.009	0.003	0.005	0.010	0.009
	Untreated 10wks	median	0.067	0.057	0.051	0.063	0.072	0.066
		IQR	0.014	0.010	0.006	0.010	0.017	0.016
	Treated 10wks	median	0.044	0.036	0.037	0.042	0.041	0.039
		IQR	0.001	0.006	0.003	0.004	0.008	0.003
	Suspended 10wks	median	0.051	0.039	0.038	0.050	0.054	0.050
		IQR	0.004	0.005	0.005	0.003	0.015	0.009
kf; 1/s	Baseline	median	1.088	1.090	0.975	1.054	1.010	0.930
		IQR	0.051	0.117	0.090	0.091	0.067	0.050
	Untreated 7wks	median	1.225	1.126	1.032	1.120	1.212	1.036
		IQR	0.135	0.031	0.054	0.036	0.054	0.114
	Treated 7wks	median	0.970	0.963	0.888	0.942	0.902	0.787
		IQR	0.129	0.180	0.089	0.085	0.113	0.049
	Untreated 10wks	median	1.253	1.402	1.262	1.322	1.274	1.208
		IQR	0.241	0.258	0.244	0.259	0.172	0.196
	Treated 10wks	median	1.058	0.863	0.790	0.984	0.906	0.787
		IQR	0.099	0.077	0.057	0.090	0.169	0.064
	Suspended 10wks	median	1.041	1.051	0.994	1.058	0.958	0.850
		IQR	0.119	0.182	0.078	0.114	0.185	0.054

T2B; μ s	Baseline	median	10.322	10.546	9.534	9.843	10.448	9.791
		IQR	1.170	0.236	0.968	0.494	0.358	0.352
	Untreated 7wks	median	9.838	10.419	9.536	9.922	10.535	9.731
		IQR	1.256	0.345	0.357	0.643	0.406	0.852
	Treated 7wks	median	9.596	9.356	9.218	9.676	9.836	9.329
		IQR	0.634	0.526	0.337	0.362	0.271	0.354
	Untreated 10wks	median	9.729	10.563	9.776	9.800	10.527	9.824
		IQR	0.056	0.036	0.233	0.348	0.097	0.123
	Treated 10wks	median	9.426	9.634	9.274	9.750	9.241	9.203
		IQR	0.294	0.593	0.328	0.179	0.328	0.187
	Suspended 10wks	median	9.695	9.692	9.515	10.017	9.892	9.584
		IQR	0.550	0.358	0.377	0.110	0.207	0.258
MTR	Baseline	median	0.372	0.391	0.336	0.381	0.404	0.361
		IQR	0.016	0.004	0.059	0.022	0.036	0.018
	Untreated 7wks	median	0.399	0.397	0.401	0.395	0.420	0.400
		IQR	0.017	0.013	0.010	0.006	0.046	0.038
	Treated 7wks	median	0.368	0.352	0.329	0.379	0.368	0.356
		IQR	0.034	0.061	0.015	0.026	0.025	0.025
	Untreated 10wks	median	0.390	0.383	0.389	0.420	0.425	0.418
		IQR	0.024	0.025	0.005	0.022	0.028	0.029
	Treated 10wks	median	0.351	0.315	0.318	0.345	0.347	0.341
		IQR	0.026	0.026	0.027	0.033	0.047	0.024
	Suspended 10wks	median	0.384	0.336	0.349	0.380	0.364	0.363
		IQR	0.011	0.025	0.025	0.027	0.019	0.018
T1obs; s	Baseline	median	1.428	1.416	1.437	1.428	1.452	1.512
		IQR	0.033	0.097	0.081	0.030	0.020	0.015
	Untreated 7wks	median	1.355	1.375	1.411	1.365	1.383	1.429
		IQR	0.085	0.034	0.043	0.049	0.083	0.102
	Treated 7wks	median	1.398	1.437	1.476	1.411	1.427	1.558
		IQR	0.045	0.092	0.126	0.091	0.155	0.087
	Untreated 10wks	median	1.275	1.326	1.338	1.302	1.346	1.422
		IQR	0.073	0.045	0.059	0.085	0.067	0.079
	Treated 10wks	median	1.382	1.486	1.530	1.420	1.465	1.527
		IQR	0.046	0.043	0.065	0.072	0.084	0.034
	Suspended 10wks	median	1.372	1.445	1.407	1.398	1.446	1.533
		IQR	0.141	0.075	0.058	0.133	0.237	0.134

SJL/J			Caudal section					
			M	I	L	M	I	L
F	Baseline	median	0.057	0.043	0.046	0.057	0.050	0.053
		IQR	0.009	0.007	0.008	0.001	0.005	0.012
	Untreated 7wks	median	0.062	0.046	0.049	0.065	0.053	0.053
		IQR	0.001	0.009	0.005	0.008	0.007	0.002
	Treated 7wks	median	0.044	0.040	0.040	0.050	0.046	0.045
		IQR	0.009	0.003	0.003	0.010	0.010	0.004
	Untreated 10wks	median	0.062	0.058	0.050	0.065	0.058	0.060
		IQR	0.013	0.003	0.006	0.012	0.010	0.009
	Treated 10wks	median	0.039	0.034	0.033	0.038	0.039	0.043
		IQR	0.005	0.001	0.004	0.004	0.003	0.002
	Suspended 10wks	median	0.051	0.043	0.044	0.051	0.044	0.046
		IQR	0.001	0.002	0.013	0.002	0.003	0.007
kf; 1/s	Baseline	median	1.027	0.881	0.784	1.088	0.965	0.932
		IQR	0.045	0.068	0.107	0.069	0.042	0.086
	Untreated 7wks	median	1.374	1.105	1.028	1.257	1.074	0.880
		IQR	0.090	0.016	0.160	0.054	0.039	0.009
	Treated 7wks	median	0.886	0.901	0.849	0.931	0.875	0.778
		IQR	0.125	0.075	0.031	0.098	0.054	0.052
	Untreated 10wks	median	1.365	1.124	1.030	1.360	1.146	0.995
		IQR	0.272	0.234	0.241	0.249	0.247	0.158
	Treated 10wks	median	0.884	0.834	0.761	0.899	0.819	0.737
		IQR	0.092	0.056	0.052	0.084	0.054	0.075
	Suspended 10wks	median	1.034	0.969	0.899	0.953	0.874	0.762
		IQR	0.075	0.030	0.119	0.029	0.029	0.061
T2B; μ s	Baseline	median	9.863	9.805	9.850	9.850	10.090	10.019
		IQR	0.339	0.456	0.304	0.157	0.201	0.582
	Untreated 7wks	median	10.339	10.188	10.010	10.183	10.312	10.287
		IQR	0.424	0.169	0.152	0.288	0.385	0.298
	Treated 7wks	median	9.559	9.582	9.335	9.426	9.891	9.597
		IQR	0.426	0.400	0.527	0.253	0.431	0.577
	Untreated 10wks	median	10.231	10.199	9.977	10.232	10.537	10.124
		IQR	0.163	0.205	0.272	0.126	0.146	0.199
	Treated 10wks	median	9.333	9.645	9.384	9.522	9.487	9.302
		IQR	0.192	0.326	0.152	0.102	0.353	0.590
	Suspended 10wks	median	9.823	9.808	9.336	9.773	9.814	9.847
		IQR	0.323	0.152	0.090	0.466	0.284	0.183

MTR	Baseline	median	0.393	0.365	0.349	0.391	0.376	0.382
		IQR	0.009	0.015	0.015	0.015	0.024	0.014
	Untreated 7wks	median	0.408	0.383	0.364	0.421	0.385	0.392
		IQR	0.006	0.023	0.009	0.042	0.032	0.010
	Treated 7wks	median	0.353	0.342	0.337	0.369	0.358	0.347
		IQR	0.024	0.016	0.011	0.045	0.035	0.018
	Untreated 10wks	median	0.400	0.401	0.375	0.415	0.397	0.410
		IQR	0.020	0.012	0.020	0.018	0.024	0.021
	Treated 10wks	median	0.331	0.322	0.314	0.331	0.339	0.352
		IQR	0.021	0.005	0.014	0.015	0.015	0.014
	Suspended 10wks	median	0.368	0.342	0.340	0.362	0.367	0.369
		IQR	0.010	0.011	0.038	0.028	0.004	0.026
T1obs; s	Baseline	median	1.454	1.564	1.627	1.434	1.534	1.553
		IQR	0.103	0.074	0.039	0.060	0.084	0.127
	Untreated 7wks	median	1.379	1.469	1.475	1.392	1.453	1.531
		IQR	0.065	0.044	0.030	0.043	0.109	0.062
	Treated 7wks	median	1.433	1.499	1.510	1.430	1.465	1.522
		IQR	0.119	0.065	0.063	0.060	0.129	0.085
	Untreated 10wks	median	1.356	1.360	1.427	1.322	1.399	1.408
		IQR	0.092	0.069	0.100	0.084	0.076	0.038
	Treated 10wks	median	1.479	1.543	1.572	1.496	1.556	1.610
		IQR	0.078	0.071	0.082	0.158	0.071	0.059
	Suspended 10wks	median	1.402	1.401	1.434	1.420	1.527	1.550
		IQR	0.017	0.051	0.165	0.043	0.024	0.085

Supplementary Table 1. Measurements of pool size ratio F , forward exchange rate k_f , transverse relaxation time of the macromolecular pool T_{2B} , observed longitudinal relaxation time T_{1obs} and magnetization transfer ratio (MTR) for C57BL/6 male mice and SJL/J female mice, reported separately for each ROI on CC (M, I, L denote medial, intermediate and lateral CC locations, respectively) and slice (rostral/caudal). The statistics were calculated from the median of values from all mice and both raters; IQR denotes interquartile range.

References

- [1] Ge Y. Multiple sclerosis: the role of MR imaging. *AJNR Am J Neuroradiol.* 2006; 27(6):1165-76.
- [2] Henkelman RM, Huang X, Xiang QS, Stanisz GJ, Swanson SD, Bronskill MJ. Quantitative interpretation of magnetization transfer. *MagnReson Med.* 1993; 29:759-66.
- [3] Quantitative MRI of the Brain: Measuring Changes Caused by Disease, Tofts P, John Wiley & Sons, Hoboken NJ, USA, 2003.
- [4] Cercignani M, Barker GJ. A comparison between equations describing in vivo MT: the effects of noise and sequence parameters. *J MagnReson.* 2008; 191(2):171-83.
- [5] McCreary CR, Bjarnason TA, Skihar V, Mitchell JR, Yong VW, Dunn JF. Multiexponential T2 and magnetization transfer MRI of demyelination and remyelination in murine spinal cord. *Neuroimage.* 2009; 45(4):1173-82.
- [6] Dula AN, Gochberg DF, Valentine HL, Valentine WM, Does MD. Multiexponential T2, magnetization transfer, and quantitative histology in white matter tracts of rat spinal cord. *MagnReson Med.* 2010; 63(4):902-9.

- [7] Levesque IR, Giacomini PS, Narayanan S, Ribeiro LT, Sled JG, Arnold DL, Pike GB. Quantitative magnetization transfer and myelin water imaging of the evolution of acute multiple sclerosis lesions. *MagnReson Med*. 2010; 63:633-40.
- [8] Falangola MF, Guilfoyle DN, Tabesh A, Hui ES, Nie X, Jensen JH, Gerum SV, Hu C, LaFrancois J, Collins HR, Helpert JA. Histological correlation of diffusional kurtosis and white matter modeling metrics in cuprizone-induced corpus callosum demyelination. *NMR Biomed*. 2014; 27(8):948-57.
- [9] Cercignani M, Basile B, Spanò B, Comanducci G, Fasano F, Caltagirone C, Nocentini U, Bozzali M. Investigation of quantitative magnetisation transfer parameters of lesions and normal appearing white matter in multiple sclerosis. *NMR Biomed*. 2009; 22:646-53.
- [10] Schmierer K, Tozer DJ, Scaravilli F, Altmann DR, Barker GJ, Tofts PS, Miller DH. Quantitative magnetization transfer imaging in postmortem multiple sclerosis brain. *J MagnReson Imaging*. 2007;26(1):41-51.
- [11] Rausch M, Tofts P, Lervik P, Walmsley A, Mir A, Schubart A, Seabrook T. Characterization of white matter damage in animal models of multiple sclerosis by magnetization transfer ratio and quantitative mapping of the apparent bound proton fraction f . *MultScler*. 2009; 15(1):16-27.

- [12] Underhill HR, Rostomily RC, Mikheev AM, Yuan C, Yarnykh VL. Fast bound pool fraction imaging of the in vivo rat brain: association with myelin content and validation in the C6 glioma model. *Neuroimage*. 2011; 54(3):2052-65.
- [13] van der Star BJ, Vogel DY, Kipp M, Puentes F, Baker D, Amor S. In vitro and in vivo models of multiple sclerosis. *CNS Neuro Disord Drug Targets*. 2012; 11(5):570-88.
- [14] Thiessen JD, Zhang Y, Zhang H, Wang L, Buist R, Del Bigio MR, Kong J, Li XM, Martin M. Quantitative MRI and ultrastructural examination of the cuprizone mouse model of demyelination. *NMR Biomed*. 2013; 26(11):1562-81.
- [15] Janve VA, Zu Z, Yao SY, Li K, Zhang FL, Wilson KJ, Ou X, Does MD, Subramaniam S, Gochberg DF. The radial diffusivity and magnetization transfer pool size ratio are sensitive markers for demyelination in a rat model of type III multiple sclerosis (MS) lesions. *Neuroimage*. 2013;74:298-305.
- [16] Ou X, Sun SW, Liang HF, Song SK, Gochberg DF. The MT pool size ratio and the DTI radial diffusivity may reflect the myelination in shiverer and control mice. *NMR Biomed*. 2009; 22:480-7.
- [17] Ou X, Sun SW, Liang HF, Song SK, Gochberg DF. Quantitative magnetization transfer measured pool-size ratio reflects optic nerve myelin content in ex vivo mice. *MagnReson Med*. 2009; 61:364-71.

- [18] Matsushima GK and Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol* 2001; 11:107-116.
- [19] Taylor LC, Gilmore W and Matsushima GK. SJL mice exposed to cuprizone intoxication reveal strain and gender pattern differences in demyelination. *Brain Pathol* 2009; 19:467-479.
- [20] Ramani A, Dalton C, Miller DH, Tofts PS, Barker GJ. Precise estimate of fundamental in-vivo MT parameters in human brain in clinically feasible time. *Magn Reson Imaging* 2002; 20:721-731.
- [21] Colombo E, Cordiglieri C, Melli G, Newcombe J, Krumbholz M, Parada L, Medico E, Hohlfeld R, Meinl E, Farina C. Stimulation of the neurotrophin receptor TrkB on astrocytes drives nitric oxide production and neurodegeneration. *J Exp Med* 2009; 3:521-535.
- [22] Schneider CA, Rasband WS, Eliceiri KW, NIH Image to ImageJ: 25 years of image analysis, *Nature Methods* 9, 671-675, 2012.
- [23] Ludwin SK. Central nervous system demyelination and remyelination in the mouse: an ultrastructural study of cuprizone toxicity. *Lab Invest* 1978; 39:597-612.

- [24] Stidworthy MF, Genoud S, Suter U, Mantei N, Franklin RJM. Quantifying the early stages of remyelination following cuprizone-induced demyelination. *Brain Pathol* 2003;13:329-339.
- [25] Fjær S, Bø L, Lundervold A, Myhr KM, Pavlin T, Torkildsen O, Wergeland S. Deep gray matter demyelination detected by magnetization transfer ratio in the cuprizone model. *PLoS One* 2013; 12: e84162.
- [26] Tsunoda I, Fujinami RS. Two models for multiple sclerosis: experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus. *J NeuropatholExp Neurol.* 1996;55(6):673-86.
- [27] McMahon EJ, Suzuki K, Matsushima GK. Peripheral macrophage recruitment in cuprizone-induced CNS demyelination despite an intact blood-brain barrier. *JNeuroimmunol.* 2002; 130:32-45.
- [28] Irvine KA, Blakemore WF. Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. *J Neuroimmunol* 2006; 175: 69-76.
- [29] Sun SW, Liang HF, Trinkaus K, Cross AH, Armstrong RC, Song SK. Noninvasive detection of cuprizone induced axonal damage and demyelination in the mouse corpus callosum. *MagnReson Med* 2006; 55:302-308.

- [30] Garcia M, Gloor M, Radue EW, StippichCh, Wetzel SG, Scheffler K, Bieri O. Fast high-resolution brain imaging with balanced SSFP: Interpretation of quantitative magnetization transfer towards simple MTR. *Neuroimage*. 2012;59(1):202-11.
- [31] Mougín OE, Coxon RC, Pitiot A, Gowland PA. Magnetization transfer phenomenon in the human brain at 7 T. *Neuroimage*. 2010; 49(1):272-81.
- [32] Cercignani M, Symms MR, Schmierer K, Boulby PA, Tozer DJ, Ron M, Tofts PS, Barker GJ. Three-dimensional quantitative magnetisation transfer imaging of the human brain. *Neuroimage*. 2005; 27(2):436-41.
- [33] Giulietti G, Bozzali M, Figura V, Spanò Barbara, Perri R, Marra C, et al. Quantitative magnetization transfer provides information complementary to grey matter atrophy in Alzheimer's disease brains. *NeuroImage* 2012; 59: 1114–22.
- [34] Savaskan NE, Weinmann O, Heimrich B, Eyupoglu IY. High resolution neurochemical gold staining method for myelin in peripheral and central nervous system at the light- and electron-microscopic level. *Cell Tissue Res*. 2009; 337:213-221.
- [35] Zhang H, Jarjour AA, Boyd A, Williams A. Central nervous system remyelination in culture- A tool for multiple sclerosis research. *Exp neurol*. 2011; 230:138-148.

- [36] Tsiperson V, Gruber RC, Goldberg MF, Jordan A, Weinger JG, Macian F, Shafit-Zagardo B. Suppression of inflammatory responses during MOG-induced experimental autoimmune encephalomyelitis is regulated by AKT3 signaling. *J Immunol*. 2013; 190:1528-1539.
- [37] Steelman AJ, Thompson JP, Li J. Demyelination and remyelination in anatomically distinct regions of the corpus callosum following cuprizone intoxication. *Neurosci Res*. 2012 ;72(1):32-42.
- [38] Xie M, Tobin JE, Budde MD, Chen CI, Trinkaus K, Cross AH, McDaniel DP, Song SK, Armstrong RC. Rostrocaudal analysis of corpus callosum demyelination and axon damage across disease stages refines diffusion tensor imaging correlations with pathological features. *J NeuropatholExp Neurol*. 2010; 69(7):704-16.
- [39] Hajnal JV, Baudouin CJ, Oatridge A, Young IR, Bydder GM. Design and implementation of magnetization transfer pulse sequences for clinical use. *J Comput Assist Tomogr*. 1992; 16(1):7-18.
- [40] Portnoy S, Stanis GJ, Modeling pulsed magnetization transfer, *MagnReson Med*2007; 58: 144–155.

CHAPTER 3

3.1.1 Summary and Conclusions

MS is one of the most common diseases of the CNS (2.1 million people suffer of MS worldwide) that mainly affects young women. This pathology is disabling, its aetiology is still elusive and its clinical manifestations are highly heterogeneous (World Health Organization, 2008). In order to provide a timely diagnosis, quantitative MRI techniques, together with other analysis, such as visually evoked potential test and CSF oligoclonal band screening, represent a useful tool for clinicians (Compost and Coles, 2000). MRI allows the evaluation of response to therapeutical intervention in clinical practice and MS longitudinal follow-up (Comi et al., 2001). Moreover, MRI studies in preclinical research allow characterization of the progression of the CNS pathology in the experimental animals.

In our study, we characterized in the cuprizone mouse model the correlation between the qMT parameter, macromolecular pool size ratio (F), measured in vivo at 7.0 Tesla, and ex vivo histological myelin analysis. In this experimental MS model, young rodents fed with cuprizone (for 5/7 weeks) develop demyelination in a reproducible manner, with well-known time and space windows and limited secondary effects, such as inflammation, oedema or toxicity, in comparison to other models, such as EAE. The reversion to normal chow diet allows remyelination, whereas the prolonged cuprizone administration (up 10 weeks or more) may suppress remyelination whilst enabling demyelination persistence (Matsushima and Morell,

2001). In particular, we investigated the events of demyelination and remyelination at the level of corpus callosum in two murine strains commonly used in MS experimental research: SJL/J and C57BL/6. SJL/J mice are susceptible to relapsing-remitting EAE and to TMEV-IDD (Tsunoda and Fujinami, 1996) but less responsive to cuprizone intoxication (Taylor et al., 2009); C57BL/6 are more widely represented in the cuprizone literature (Matsushima and Morell, 2001; Ou et al., 2009a; Ou et al., 2009b; Taylor et al., 2010).

In the present study, F parameter has been shown to have superior sensitivity to evaluate in vivo cuprizone-induced demyelination and remyelination than other MRI parameters, such as k_f , T_{2b} , T_{1obs} or MTR. Moreover, F is preferable since it has a specific relevance to myelin concentration. In our study, F directly linked to myelin concentration showed higher sensitivity in detecting cuprizone-induced demyelination and remyelination than k_f , T_{2b} , T_{1obs} or MTR. MTR is a relatively unspecific parameter, since it is influenced not only by the pool size ratio but also by the exchange rate and liquid pool relaxation time. Moreover, the biological interpretation of k_f and T_{2b} is less clear; there is some evidence that k_f may be related to metabolic changes, and that T_{2b} , which generally shows small and inconsistent changes in pathology, may be influenced by fiber orientation (Cercignani et al., 2005; Cercignani et al., 2009; Giulietti et al., 2012). Our finding of significant F value reduction during cuprizone treatment and recovery after cuprizone suspension, together with significant correlation with histological measurements of myelin content, corroborates previous ex vivo imaging results on shiverer mice demonstrating that this quantitative parameter is linked to myelin

status (Ou et al., 2009a; Ou et al., 2009b). Indeed, our data (both F value and quantitative histological analysis) showed a significant demyelination during the initial period of treatment (5/7 weeks) followed by aggravation for continued treatment at 10 weeks in both strains; spontaneous remyelination (consequent to cuprizone withdrawal) was evident in C57BL/6 mice. While histology with black gold II confirmed this pattern, significant remyelination was detected with MBP immunofluorescence only for SJL/J mice. This discrepancy may be related to the different cuprizone-susceptibility of the strains (Taylor et al., 2009; Taylor et al., 2010). Demyelination was accompanied by a significant reduction in β -tubulin levels only in C57BL/6 mice. Previous studies demonstrated that axonal damage and neuronal loss follow demyelination in the corpus callosum when treatment is protracted; this may not be a direct result of cuprizone toxicity, but a consequence of the physiological reliance of neurons to oligodendrocyte support (Matsushima and Morell, 2001; Irvine and Blakemore, 2006; Sun et al., 2006). On the contrary, β -tubulin levels of SJL/J mice remained clearly unaffected by cuprizone demyelination. Moreover, significant correlations between F and histological measurements were found. Additionally, due to the small sample size we only considered rank-order correlation and the exact transfer function, i.e. the linearity of the relationship between parameters, remains to be confirmed.

In summary, our study concludes that F variations are primarily and selectively driven by myelin degradation, not by neuronal loss. Using a 7.0 Tesla MRI scan, we developed a protocol able to analyse two brain section in an overall scan time of ~ 2 hours, including pilot

sequences, positioning and repeated series. This method is better suitable for in vivo imaging in animals, compared to previous published protocols (such as Janve et al., 2013). Demonstration of accurate measurement of myelin status in vivo is particularly important, as the main motivation for using quantitative magnetization transfer in preclinical studies is the possibility to perform longitudinal measurements, reducing the number of animals.

Finally, our study demonstrated potential practical implications at two levels. First, it reaffirms the sensitivity and biological specificity of F measurements, suggesting that qMT-MRI should be used as a key outcome measure in trials of MS therapeutics. Second, it demonstrates that qMT-MRI can be efficiently implemented in vivo on a small-animal scanner and that its sensitivity to myelin changes is comparable to histological measures, prompting its consideration as a primary investigational tool in preclinical research on murine models of MS.

3.1.2 Future Perspectives

In order to establish the power of qMT imaging technique, we want to apply the MRI sequences to other experimental demyelinating models, showing different types of lesions. In contrast to cuprizone model, EAE and TMEV-IDD models are characterized by great ranges of immune infiltrates (Sun and Wekerle, 1996; Vanderlugt and Miller, 2002) and may be of help in ruling out the effects of inflammation on qMT measures. Indeed, at present we cannot exclude a confounding effect of inflammatory changes on F measures and further

investigations are necessary to specifically address the question. Moreover, starting from the qMT protocol for myelin imaging in brain, we will develop a protocol suitable for myelin imaging in spinal cord, as this is the target CNS compartment in EAE and TMEV-IDD models. The two major critical points for the correct implementation of the methodology are the size of spinal cord transversal sections and the preferential orientation of fibers. Moreover, it would be important to clarify the biological meaning of qMT parameters (i.e. k_f and T_{2b}) to further characterize the demyelinating lesions. In order to achieve this goal it will be necessary to monitor the variations of all qMT parameters in other types of lesions and before/after treatment.

One of the main reasons to monitor the myelin status using MRI techniques instead of histology is the prospect to reduce the number of animals needed for preclinical research. Unfortunately at present, qMT-MRI cannot completely replace histology altogether, not only because of the spatial resolution limitation of MRI, but also because F measurements are unavoidably affected by factors other than myelin density due to a simplistic model (two-pools are only an approximation) and limitations such as T_1 -weighting effects, inhomogeneous B_1 , error in the attained excitation angles due to non-linearity etc. and measurement noise (Ramani et al., 2002; Tofts, 2003; Cercignani and Barker, 2008).

In recent years, qMT has emerged as a non-conventional MRI technique potentially helpful to accurately characterize newly forming lesions and the dissemination of lesions in time and space (Levesque et al., 2010; Bonnier et al, 2014). We hope that our study, supporting

the specificity of qMT to detect myelin status, may help future translation of the technique as common MRI diagnosis tool.

3.1.3 References

Bonnier G, Roche A, Romascano D, Simioni S, Meskaldji D, Rotzinger D, Lin YC, Menegaz G, Schluep M, Du Pasquier R, Sumpf TJ, Frahm J, Thiran JP, Krueger G, Granziera C. Advanced MRI unravels the nature of tissue alterations in early multiple sclerosis. *Ann Clin Transl Neurol*. 2014 Jun;1(6):423-32.

Cercignani M, Symms MR, Schmierer K, Boulby PA, Tozer DJ, Ron M, Tofts PS, Barker GJ. Three-dimensional quantitative magnetisation transfer imaging of the human brain. *Neuroimage*. 2005; 27(2):436-41.

Cercignani M, Barker GJ. A comparison between equations describing in vivo MT: the effects of noise and sequence parameters. *J MagnReson*. 2008; 191(2):171-83.

Cercignani M, Basile B, Spanò B, Comanducci G, Fasano F, Caltagirone C, Nocentini U, Bozzali M. Investigation of quantitative magnetisation transfer parameters of lesions and normal appearing white matter in multiple sclerosis. *NMR Biomed*. 2009; 22:646-53.

Comi G, Rovaris M, Leocani L, Martinelli V, Filippi M. Clinical and MRI assessment of brain damage in MS. *Neurol Sci*. 2001 Nov;22 Suppl 2:S123-7.

Compston A, Coles A. Multiple sclerosis. *Lancet*. 2002 Apr 6;359(9313):1221-31.

Giulietti G, Bozzali M, Figura V, Spanò Barbara, Perri R, Marra C, et al. Quantitative magnetization transfer provides information complementary to grey matter atrophy in Alzheimer's disease brains. *NeuroImage* 2012; 59: 1114–22.

Irvine KA, Blakemore WF. Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. *J Neuroimmunol* 2006; 175: 69-76.

Janve VA, Zu Z, Yao SY, Li K, Zhang FL, Wilson KJ, Ou X, Does MD, Subramaniam S, Gochberg DF. The radial diffusivity and magnetization transfer pool size ratio are sensitive markers for demyelination in a rat model of type III multiple sclerosis (MS) lesions. *Neuroimage*. 2013;74:298-305.

Levesque IR, Giacomini PS, Narayanan S, Ribeiro LT, Sled JG, Arnold DL, Pike GB. Quantitative magnetization transfer and myelin water imaging of the evolution of acute multiple sclerosis lesions. *Magn Reson Med*. 2010 Mar;63(3):633-40.

Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol*. 2001 Jan;11(1):107-16.

Ou X, Sun SW, Liang HF, Song SK, Gochberg DF. The MT pool size ratio and the DTI radial diffusivity may reflect the myelination in shiverer and control mice. *NMR Biomed.* 2009a; 22:480-7.

Ou X, Sun SW, Liang HF, Song SK, Gochberg DF. Quantitative magnetization transfer measured pool-size ratio reflects optic nerve myelin content in ex vivo mice. *MagnReson Med.* 2009b; 61:364-71.

Ramani A, Dalton C, Miller DH, Tofts PS, Barker GJ. Precise estimate of fundamental in-vivo MT parameters in human brain in clinically feasible time. *MagnReson Imaging* 2002; 20:721–731.

Sun D, Wekerle H. Ia-restricted encephalitogenic T lymphocytes mediating EAE lyse autoantigen-presenting astrocytes. *Nature.* 1986 Mar 6-12;320(6057):70-2.

Sun SW, Liang HF, Trinkaus K, Cross AH, Armstrong RC, Song SK. Noninvasive detection of cuprizone induced axonal damage and demyelination in the mouse corpus callosum. *MagnReson Med* 2006; 55:302-308.

Taylor LC, Gilmore W, Matsushima GK. SJL mice exposed to cuprizone intoxication reveal strain and gender pattern differences in demyelination. *Brain Pathol.* 2009 Jul;19(3):467-79.

Taylor LC, Gilmore W, Ting JP, Matsushima GK. Cuprizone induces similar demyelination in male and female C57BL/6 mice and results

in disruption of the estrous cycle. *J Neurosci Res.* 2010 Feb 1;88(2):391-402.

Tofts P. Quantitative MRI of the brain. Measuring changes caused by disease. Wiley. 2003.

Tsunoda I, Fujinami RS. Two models for multiple sclerosis: experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus. *J NeuropatholExp Neurol.* 1996;55(6):673-86.

Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol.* 2002 Feb;2(2):85-95.

World Health Organization: Atlas multiple sclerosis resources in the world 2008. Geneva: WHO Press; 2008.

Publications

In vivo quantitative magnetization transfer imaging correlates with histology during de- and re-myelination in cuprizone-treated mice

Paper Submitted to NMR in Biomedicine

Laura Turati¹, MSc; Marco Moscatelli¹, MD; Alfonso Mastropietro², PhD; Nicholas G Dowell³, PhD; Ileana Zucca², PhD; Alessandra Erbetta⁴, MD; Chiara Cordiglieri¹, PhD; Greta Brenna¹, MSc; Beatrice Bianchi¹, MSc; Renato Mantegazza¹, MD; Mara Cercignani³, PhD; Fulvio Baggi^{1#}, PhD; Ludovico Minati^{2,*,#}, PhDCEngCPhysCSci

¹Neuroimmunology and Neuromuscular Diseases unit, ² Scientific department and ⁴ Neuroradiology unit, Fondazione IRCCS Istituto Neurologico “Carlo Besta”, Milano, Italy;³ Brighton and Sussex Medical School, Brighton, United Kingdom;

These two authors contributed equally to study design and supervision.

Acknowledgments

This study would not have been possible without a network of scientists and colleagues.

Firstly, I would like to thank Dr. Renato Mantegazza, who believed in my skills and gave me the opportunity to be a part of Preclinical Neuroimmunology Laboratory and to carry out my PhD in his Operative Unit.

Many thanks to Dr. Fulvio Baggi, for the supervision of the project during these years, for the scientific support and for the revision of my thesis.

I wish to thank Dr. Pia Bernasconi, mentor of my PhD, who advised me during my scientific training.

Thanks to Dr Ludovico Minati for his technical MRI expertise, essential for this project.

A very special thank to all my colleagues, Dr Chiara Cordiglieri, Dr Alessandra Consonni and Dr Roberta Marolda for the scientific support and for the help they gave me during these years.

Thanks to my family that always support me and my great passion for research.