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PhD course in NEUROSCIENCE

Combining advanced MRI techniques and positron emission tomography to explore remyelination in multiple sclerosis

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Supervisor University of Padua: Prof. Paolo Gallo

Supervisor Sorbonne Université: Prof. Benedetta Bodini

Ph.D. student: LAZZAROTTO ANDREA

Alla mia Famiglia

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Summary

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) with inflammatory and degenerative components affecting both the white (WM) and the grey matter (GM). The key neuropathological hallmarks of MS include demyelination, inflammation, astrocytic gliosis, and neurodegeneration, which is considered the main pathological substrate of clinical progression. Among these, myelin loss is considered the cornerstone of the pathophysiology of MS and the dynamics of myelin content change are thought to have a relevant impact on neurodegeneration and disability all along the course of the disease. Endogenous myelin repair is a physiological response to a demyelinating event and is mediated by population of adult brain resident progenitor cells which migrate into areas of demyelination and differentiate into actively myelinating oligodendrocytes. In experimental models a high variability in the extent of remyelination has been observed in MS patients, with myelin repair being widespread in few patients only, and sparse or ineffective in the others. Several factors have been suggested to influence the efficacy of this process and even in the same patient, a high heterogeneity may exist between different areas, suggesting the presence of local factors influencing the extent of remyelination.

Although our knowledge concerning the biologic process of myelin repair has progressively increased over time, to date its clinical impact remains partly unknown. The exploration of myelin content change in MS patients in vivo has been challenging, due to the heterogeneity of the disease but also to the lack of appropriate methodologies to study the complex interplay between the pathological changes occurring both to the myelin sheet and to the neuroaxonal unit, particularly at the cortical level. Various methods have been proposed for this purpose, among which [^{11}C]PIB-PET has been proved sensitive and specific in the evaluation of myelin content. However, the reduced spatial resolution, together with the fact that a reference region, represented by the cerebral cortex, is required for signal quantification, limit the application of this method to cortical grey matter. Among the various alternative techniques to the PET imaging, magnetization transfer ratio (MTR), an imaging metric sensitive to myelin content changes in MS, has been shown to be lower in cortical lesions than in normally myelinated cortex and has been successfully employed to generate maps sensitive to myelin loss in cortical grey matter.

In this context, the aims of my PhD project were focused on the exploration of myelin repair, focusing primarily on the delineation of a method capable of producing patient-specific cortical maps of demyelination and remyelination using MTR. With these maps I had the chance to explore the spatial distribution of myelin repair and to evaluate the role of both cortical

demyelination and remyelination in determining cortical atrophy and clinical progression, outlining though the supposed role of myelin repair in preventing neurodegeneration in MS.

Résumé

La sclérose en plaques (SEP) est une maladie inflammatoire du système nerveux central (SNC) dont les composantes inflammatoires et dégénératives touchent à la fois la substance blanche et la substance grise. Les principales caractéristiques neuropathologiques de la SEP comprennent la démyélinisation, l'inflammation, la gliose astrocytaire et la neurodégénérescence, qui est considérée comme le principal substrat pathologique de la progression clinique. Parmi ceux-ci, la perte de myéline est considérée comme la pierre angulaire de la physiopathologie de la SEP et la dynamique du changement de contenu de la myéline est supposée avoir un impact pertinent sur la neurodégénérescence et le handicap tout au long de l'évolution de la maladie. La réparation endogène de la myéline est une réponse physiologique à un événement démyélinisant et est médiée par une population de cellules progénitrices résidant dans le cerveau adulte qui migrent dans les zones de démyélinisation et se différencient en oligodendrocytes myélinisants actifs. Dans les modèles expérimentaux, une grande variabilité dans l'étendue de la remyélinisation a été observée chez les patients atteints de SEP, la réparation de la myéline étant répandue chez quelques patients seulement, et éparse ou inefficace chez les autres. Plusieurs facteurs ont été suggérés pour influencer l'efficacité de ce processus et même chez un même patient, une grande hétérogénéité peut exister entre différentes zones, suggérant la présence de facteurs locaux influençant l'étendue de la remyélinisation.

Bien que nos connaissances concernant le processus biologique de réparation de la myéline aient progressivement augmenté au fil du temps, son impact clinique reste à ce jour partiellement inconnu. L'exploration des modifications du contenu de la myéline chez les patients atteints de SEP in vivo est un défi, en raison de l'hétérogénéité de la maladie mais aussi du manque de méthodologies appropriées pour étudier l'interaction complexe entre les changements pathologiques survenant à la fois au niveau de la myéline et de l'unité neuroaxonale, en particulier au niveau cortical. Diverses méthodes ont été proposées à cette fin, dont la [^{11}C]PIB TEP qui s'est avérée sensible et spécifique dans l'évaluation du contenu de la myéline. Cependant, la résolution spatiale réduite, ainsi que le fait qu'une région de référence, représentée par le cortex cérébral, est nécessaire pour la quantification du signal, limitent l'application de cette méthode à la substance grise corticale. Parmi les différentes techniques alternatives au [^{11}C]PIB TEP, le rapport de transfert de magnétisation (MTR), une mesure d'imagerie sensible aux changements du contenu en myéline dans la SEP, avec une diminution

dans les lésions corticales que dans le cortex normalement myélinisé et a été utilisé avec succès pour générer des cartes sensibles à la démyélinisation dans la substance grise corticale.

Dans ce contexte, les objectifs de mon projet de doctorat étaient axés sur l'exploration de la réparation de la myéline, en se concentrant principalement sur la délimitation d'une méthode capable de produire des cartes corticales de démyélinisation et de remyélinisation spécifiques aux patients à l'aide de la MTR. Ces cartes m'ont permis d'explorer la distribution spatiale de la réparation de la myéline et d'évaluer le rôle de la démyélinisation et de la remyélinisation corticales dans l'apparition de l'atrophie corticale et de la progression clinique, soulignant ainsi le rôle supposé de la réparation de la myéline dans la prévention de la neurodégénérescence dans la SEP.

Chapter 1- Introduction

Chapter 1.1 – Multiple sclerosis

1.1.1 Epidemiology and general overview

Multiple sclerosis (MS) is a chronic autoimmune-driven neurodegenerative disease affecting the central nervous system affecting 2.3 million people worldwide (GBD 2016 MS Collaborators, 2019). The disease remains a major public health issue as the leading cause of non-traumatic neurological disability among young adults. No cure has been found for MS.

The global prevalence of MS differs by sex, as MS is known to affect more women than men (Trojano, et al., 2012) and varies greatly across continents. Indeed, a demographic heterogeneity has been described in the prevalence of MS worldwide from high levels (>120 cases of MS/100 000 habitants) in North America and some northern European countries to lower levels (<60 cases of MS/100 000 habitants) in Asia and Sub-Saharan Africa. However, the notion of a latitude gradient has been questioned because of the heterogeneity in the diagnosis accuracy and case ascertainment between the northern and southern hemispheres (Koch-Henriksen & Soelberg Sorensen, 2011).

Of particular interest, lower circulating levels of vitamin D have been associated with a higher risk of MS (Ascherio, et al., 2014). Other environmental factors such as cigarette smoking (Ramanujam, et al., 2015), Epstein- Barr virus infection (Bjornevik, et al., 2022), organic solvent, adolescent obesity, and working night shifts have been identified (Olsson, et al., 2017).

Recently, Tateo and colleagues investigated the role of pollution in MS. They found out that MS prevalence was significantly higher in urban areas (ranging from 219 in Padua City to 169/100,000 in other urban areas) compared to isolated villages (116/100,000) or rural domains (109/100,000) and strongly correlated with the annual average concentration of PM2.5 ($r = 0.81$, $p < 0.001$). Regression analysis further associated MS cases with PM.2.5 average concentration ($\beta = 0.11$, $p < 0.001$). These results suggest that air pollutants may be one of the possible environmental risk factors for MS(Tateo, et al., 2019).

The increased heritability within family provides evidence that genetic factors have a prominent role in the development of MS. The association between MS and variations in the genes encoding human leukocyte antigens (HLA) within the major histocompatibility complex was first observed several decades ago (Jersild , et al., 1972). As an example, it has been shown that

carriers of the class II variant HLA DRB1*15:01 allele are about three times more likely to develop MS than non-carriers (Patsopoulos, et al., 2013). Not less than 110 other non-HLA genes have been identified as heritable risk factors for MS (The International Multiple Sclerosis Genetics Consortium, 2007), such as IL2RA and IL7RA. Interestingly, most of the non-HLA known MS-associated genes regulate adaptive and innate immunity, cytotoxic and regulatory T cell and microglia function, providing further evidence that MS is primarily an immune-mediated disease.

1.1.2 Diagnostic Criteria

Since the first description of clinical features of MS by Jean Martin Charcot, the diagnostic criteria have changed considerably over time (Przybek, et al., 2015). The first guidelines provided by Charcot, followed by Marburg, outlined triads of symptoms, which appeared to be not specific enough to MS and occurred in other neurological disorders (Charcot, 1868). In 1954, Allison and Milliar have classified their pathological cases “according to an arbitrary scheme”, introducing for the first time the clinical classification of patients as “early”, “probable” or “possible” MS (Allison and Millar, 1954). This classification recognized the appearance of symptoms at different time points involving different regions of the CNS, introducing for the first time the notion of dissemination in time (DIT) and dissemination in space (DIS) as essential elements to diagnose MS. The classification mentioned above were used by Schumacher and colleagues to develop new guidelines for the diagnosis of “clinically definite” form of MS and to exclude alternative diagnoses (Schumacher et al., 1965).

In 1983, a committee of MS experts have established a formalized guideline for the diagnosis of MS (Poser, et al., 1983). These were based on previous Schumacher’s criteria (Schumacher et al., 1965). To improve the diagnosis, Poser and colleagues have proposed to classify patients in two major groups, definite and probable MS (Poser, et al., 1983). The main clinical feature was a “relapse” defined as the occurrence of new neurological symptoms, typical of MS, lasting for at least 24 hours. Poser criteria allowed the diagnosis of definite MS when there were at least two separate attacks (evidence of DIT with the second attack occurring at least 30 days after the beginning of patient clinical recovery from the first attack), with clinical evidence of two separate lesions involving two different parts of the CNS (proof for DIS) or laboratory support by examination of the cerebro-spinal fluid (CSF) (presence of oligoclonal band or increased production of immunoglobulin G). CSF examination was included in these criteria to help in the differential diagnosis by providing evidence of MS through the presence of oligoclonal bands and to rule out other infectious or inflammatory conditions.

The significant development of MRI led to an update of the MS criteria in 1997 in which, for the first time, DIS and DIT could be achieved also by MRI (Barkhof, et al., 1997). Subsequently, the criteria were further updated byTintoré and colleagues (Tintoré, et al., 2000) . The criteria for the diagnosis of definite MS were then revised in 2001 by a consensus of MS experts under the supervision of Ian McDonald (McDonald, et al., 2001), revised in 2005 (Polman, et al., 2005), 2011 (Polman, et al., 2011) and 2017 (Thompson, et al., 2018). These successive revisions aimed to simplify the diagnostic criteria, increasing sensitivity without modifying the specificity, and thus allowing an earlier diagnosis. Based on the McDonald criteria, a diagnosis of MS can be reached on clinical assessment alone if the patient has experienced two or more attacks associated to clinical evidence of at least two lesions in the CNS. In other cases (two attacks with evidence of a single lesion, one attack, progressive course), MRI should be used to assess either DIS or DIT always coupled with the CFS analysis: DIS can be demonstrated with at least one lesion in two typical regions (periventricular, Juxtacortical/cortical, infratentorial and spinal cord) and DIT can be achieved by the simultaneous presence of gadolinium-enhancing and not gadolinium-enhancing lesions, by the appearance of new lesions over time and/or by the presence of unique CSF oligoclonal bands. The first demyelinating attack not fulfilling the current diagnostic criteria for MS (absence of DIT and or DIS), defines a so-called clinically isolated syndrome (CIS).

1.1.3 MS clinical subtypes and treatments

The large clinical heterogeneity of MS has been conventionally simplified into three main forms (Filippi, et al., 2018).

In most cases, episodic and reversible phases of neurological deficits lasting at least 24 hours and up to several weeks - the so-called relapses - characterize the most common form of the disease, or relapsing-remitting MS (RRMS). Over the course of RRMS, and with the repetition of relapses in the absence of therapeutic intervention, the natural history of the disease leads to the development of permanent neurological deficits and disability progression in a median time of 20 years (range 1–51 years), defining the so-called secondary progressive MS (SPMS) (Cree, et al., 2021). A small number of individuals (around 10-12%), usually older at disease onset and with equal proportion of men and women, exhibit a more insidious progressive course, with gradually increasing neurological disability from onset, referred to as primary progressive MS (PPMS) (Miller & Leary, 2007). Besides the three typical forms (RRMS, SPMS, PPMS), the National Multiple Sclerosis Society Advisory Committee on Clinical Trials in MS defined a progressive relapsing MS (PRMS) as the subtype with progressive disease from the onset, but

with acute relapses (Lublin & Reingold, 1996). Finally, within each subgroup, the disease can be further classified as active or not active, based on the presence of clinical of neuroimaging activity. In the case of progressive MS, for instance, we can distinguish an active disease with/without progression, or a non-active disease with/without progression (the latter corresponding to a stable MS) (Lublin, et al., 2014)(Figure 1)

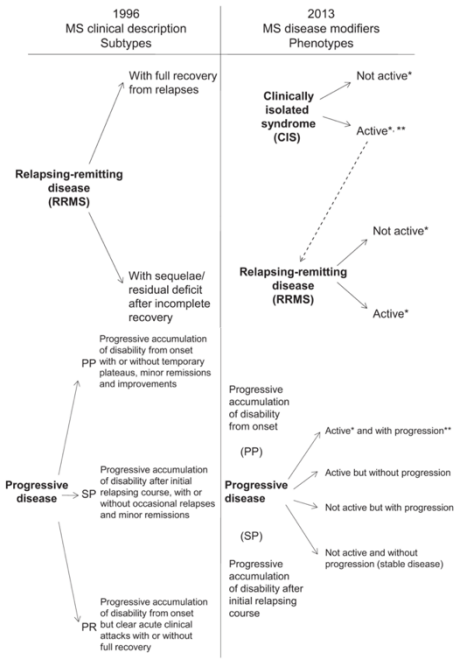


Figure 1. The 1996 vs 2013 MS phenotype descriptions (Lublin et al., 2014). Activity is determined by clinical relapses and/or MRI activity (contrast-enhancing lesions; new or unequivocally enlarging T2 lesions assessed at least annually) (Lublin et al., 2014).

MS is one of the few neurological disorders where the introduction of disease-modifying therapies (DMTs) having anti-inflammatory, immunomodulatory or purely immunosuppressive action, has significantly changed the natural history of the disorder. Apart from the symptomatic treatment (i.e., for spasticity, urinary problems, neuropathic pain, and others), and the acute treatment of relapses with i.v. ororal steroids, or in some cases plasma-exchange, the core of long-term MS treatment is based on DMTs, which reduces the risk of new lesion formation and significantly lowers the annual relapse rate. These drugs, classically distinguished in first- (Glatiramer Acetate, Interferons, Teriflunomide, Dimethylfumarate, Diroximelfumarate) and second-line (Fingolimod, Natalizumab, Ocrelizumab, Ofatumumab, Rituximab, Alemtuzumab) treatments based on their efficacy and risk profile, may act in several ways: by modulating cytokine secretion, trapping pro-inflammatory cells in lymph nodes and/or blocking their access to the CNS , reducing immune cell proliferation, or lowering the levels of specific immune cell

subsets through a selective targeting (Tintore, et al., 2019). The latter strategy has seen a huge expansion in the last few years thanks to the introduction of monoclonal antibodies directed against CD20+ (Rituximab, Ocrelizumab, Ofatumumab) or CD52+ (Alemtuzumab) cells. Additionally, other compounds with old or novel mechanisms of action are currently being developed or approaching the final approval phase (e.g., Bruton's tyrosine kinase [BTK] inhibitors, Ponesimod, Ozanimod, Ibudilast) (Cree, et al., 2019).

Current therapies for MS are very effective in targeting the immune system and in preventing clinical relapses but fail to prevent or delay neuro-axonal degeneration and, as a result, clinical progression (Ontaneda, et al., 2015).

Chapter 1.2 – Myelin loss and repair as key elements in the pathogenesis of MS

Multiple sclerosis is traditionally considered as a chronic inflammatory - demyelinating disease of the CNS, which leads to the formation of demyelinated plaques with different degrees of axonal preservation and reactive astrocytic scar formation. These basic hallmarks of MS pathology were defined during the 19th century, starting with the macroscopic drawings of brain and spinal cord alterations by (Carswell, 1838) (Cruveilhier, 1842). Following these descriptions, Charcot was the first to define MS as a focal inflammatory demyelinating disease of the white matter, and the first to accurately identify its principal pathological hallmarks (Charcot, 1868). For some time, MS has been considered a neurological disease exclusively resulting from focal demyelinated plaques in the white matter. Later it became clear that lesions are also present in the gray matter, including the cortex, the deep GM, and the GM of the spinal cord (Brownell & Hughes, 1962). Furthermore, a progressive process of neurodegeneration which affects the brain and spinal cord has been shown to develop since the earliest phases of the disease, independently of the clinical form (Borgström, et al., 2020) (Margoni, et al., 2021; Lazzarotto, et al., 2021).

Since the first characterization, the general pathology of MS has been well described and the pathological hallmarks of MS include now inflammation, demyelination, failure of remyelination, gliosis and neurodegeneration, which affect the WM and the GM both locally and diffusely (Haider, et al., 2016) (Lassmann, 2018).

1.2.1 White matter inflammation and myelin loss

White matter inflammation in MS involves both the adaptive (T and B cells, in the periphery and in the CNS) and the innate immune system (microglia) (Dendrou, et al., 2015) (Bar-Or & Li, 2021). Historically, the key role of T cells was supported by animal models of MS, particularly the experimental autoimmune encephalomyelitis (EAE) mice, in which the transfer from affected to healthy animals of aberrantly activated or deregulated pro-inflammatory effector T cells was able to induce demyelination, neuro-axonal injury and relapse in the recipients. Experimental data support the primary contribution of CD4+ T helper cells expressing interleukin 17 (Th17), Interferon γ-secreting CD4+ T cells (Th1 cells), and CD8+ T cells, that include the subset of CD8+ MAIT cells (Boos, et al., 1983) (Willing, et al., 2014); (Machado-Santos, et al., 2018). Pro-

inflammatory T cells in MS are activated by antigen presenting cells (APCs, such as B cells and myeloid cells), responsible of processing intact myelin antigens. In turn, differentiated pro-inflammatory effector T cells influence B cell properties by establishing a positive feedback loop of proinflammatory B and T cell development (Weber & Hemmer, 2010) (van Langelaar, et al., 2020). They also secrete cytokines that suppress Th2 differentiation, with the subsequent reduction of anti-inflammatory mediators released by this Th subtype. Under the control of activated Th cells, antigen-primed B cells can then differentiate into antibody-secreting plasma cells (responsible, for instance, of the presence of oligoclonal bands in the CNS) or become memory B cells in the germinal centers. Moreover, some B cells also have anti-inflammatory properties and secrete regulatory cytokines which foster the development of other immunomodulatory cells, such as regulatory T cells (Vasileaiadis, et al., 2018). Therefore, T cells and B cells closely support one another in the disease pathogenesis (van Langelaar, et al., 2020) (Bar-Or & Li, 2021), as confirmed by the effectiveness of therapeutic strategies targeting one or the other cell type, or both. Activated innate immune cells coming from the periphery, together with CNS-resident cells, secrete proinflammatory mediators that lead to demyelination and axonal damage. In this scenario, the pro-inflammatory phenotype of activated innate immune cells, together with astrocytes, perpetuate neurotoxic inflammation, oligodendrocyte injury and prevent the terminal differentiation of oligodendrocyte progenitor cells into myelin-generating oligodendrocytes (Filippi, et al., 2018) (Figure 2).

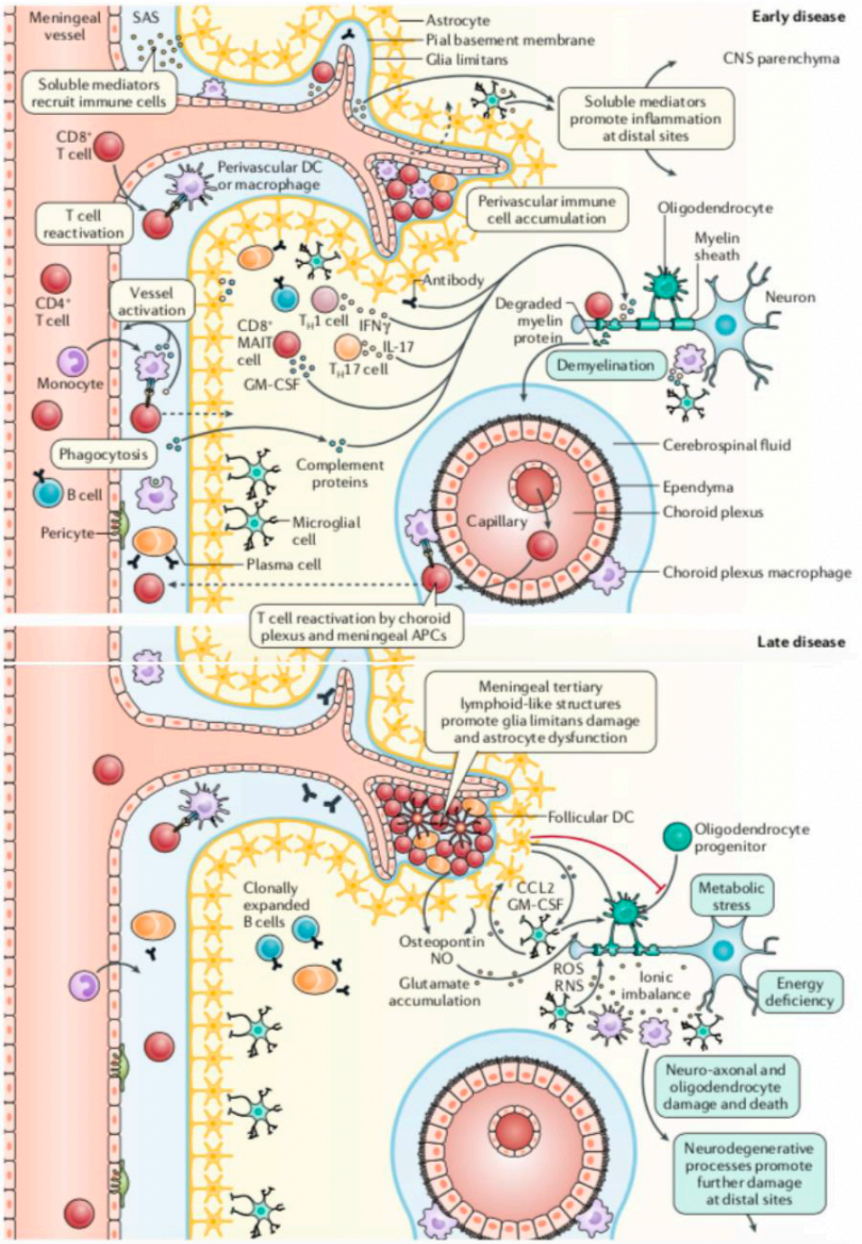


Figure 2. Immune system involvement in different stages of MS. From Filippi M, Bar-Or A, Piehl F, et al (2018). Multiple sclerosis. Nat Rev Dis Primers 4:43.

The intrinsically immune nature of MS is confirmed by histopathological studies (Lassmann, 2013). Inflammatory cells invading the CNS give rise to focal demyelinated plaques in the white matter, mainly distributed around post-capillary venules and showing a breakdown of the blood-brain barrier. Multifocal white matter zones of inflammation with blood-brain-barrier (BBB) breakdown, T-lymphocytic (CD8+ more than CD4+), B lymphocytic, macrophage infiltrations (Frischer, et al., 2009) and oligodendrocyte death result in myelin sheath destruction, formation of CNS plaques composed of several inflammatory cells and their products, demyelinated and transected axons, and reactive astrocytes. White matter plaques with these features have the

highest density of inflammatory infiltrates (Zrzavy, et al., 2017) (Machado-Santos, et al., 2018), are actively demyelinating and can be typically found in the brain of patients with RRMS. In progressive forms, the decline of inflammation in favor of an increased neurodegeneration is reflected in lesions that tend more frequently to be inactive, with less acute inflammation, more severe myelin loss, astrogliosis and peri-plaque microglial activation. Completely inactive demyelinated lesions are end-stage lesions without ongoing myelin destruction and inflammation, comparable to a scar, with very few myelin, reduced cellularity and axonal density (Kutzelnigg & Lassmann, 2014). Furthermore, patients with a long disease history and SPMS also show chronic active plaques characterized by more macrophages with myelin degradation products at the lesion edge than in the center (Frischer, et al., 2015). Progressive patients typically present plaques called "slowly expanding" or "smoldering" which show an inactive demyelinated center and activated microglia and few macrophages containing myelin debris at the lesion border, reflecting a slow ongoing demyelination at the lesion edge (Kutzelnigg & Lassmann, 2014) (Lassmann, 2019) (Stadelmann, et al., 2019). The presence of either active or slowly expanding lesions has been associated with younger age of death, shorter disease duration and more aggressive course compared with pathologically inactive forms of disease (Absinta, et al., 2019). The heterogeneity of lesion demyelination has been well captured by pathological studies, which have allowed to identify four different patterns of white matter lesions, on the basis of myelin protein loss, oligodendrocyte destruction, complement activation, and plaque morphology (Lucchinetti, et al., 2000). Two of them (I and II) are characterized by a typical perivenular distribution of lesions, along with a T-cell and macrophage-dominated inflammation, but with a more prominent role of antibodies in pattern II, reflected by the deposition of immunoglobulins and complement C9neo antigen at sites of active demyelination. The other patterns (III and IV) were highly suggestive of a primary oligodendrocyte dystrophy, with pattern III mostly found in more aggressive RRMS, and pattern IV exclusively present in a subgroup of patients with PPMS (Lucchinetti, et al., 2000).

Beside the presence of focal demyelinated plaques in the WM, the brain of patients with MS is affected by pathological changes in a more global sense. Changes in the normal-appearing white matter (NAWM) consists of a diffuse inflammatory process associated with a diffuse activation of the microglia. In addition, there is a diffuse axonal injury throughout the whole brain, which occurs independently of demyelination (Kultzelnigg, et al., 2005). In 2005, Kutzelnigg and colleagues provided a clear picture of the NAWM pathology by analyzing 52 post-mortem brain samples of patients with different clinical courses (11 acute MS, 6 RRMS, 20 SPMS, and 14 PPMS). They described a global reduction in the intensity of myelin staining due to decreased

fiber density as well as diffuse axonal injury that is more significant in progressive patients compared with RRMS or patients with acute MS. Axonal injury and loss are visible by the presence of focal axonal swelling, axonal end bulbs and degenerating axons throughout the WM. Axonal degeneration affects the whole WM, particularly around demyelinated plaques and in defined fiber tracts emerging from WM lesions.

Diffuse changes in NAWM also occur in presence of inflammatory infiltrates, mainly composed of CD8+ T-lymphocytes associated with profound microglial activation, which are more present in patients with progressive disease compared to RRMS (Kultzelnigg, et al., 2005). An interesting result of the study from Kutzelnigg and colleagues was the absence of correlation between focal WM lesion load and microglial activation or axonal injury in the NAWM, suggesting that diffuse WM injury can develop independently of demyelinating WM lesions. Moreover, diffuse WM injury appeared to be correlated with the extent of cortical demyelination. This result suggests that neurodegeneration in cortical plaques could have a stronger impact on WM injury than WM lesions, through anterograde degeneration, or that cortical lesions appear predominantly in area connected to WM damages, through retrograde degeneration (Calabrese, et al., 2015).

1.2.2 Cortical inflammation and myelin loss

Despite having been historically considered a WM disease, it is now clear that MS is also characterized by a significant involvement of the GM (Brownell & Hughes, 1962).

While the focal bulk invasion of T- and B-lymphocytes with profound blood brain barrier leakage predominately affects the white matter giving rise to the classical WM active demyelinated plaques, a slow accumulation of T-cells and B-cells in the absence of major blood brain barrier damage has been observed in the connective tissue spaces of the brain, such as the meninges and the large perivascular Virchow Robin spaces, where T- and B-cells form aggregates or in most severe cases structures in part resembling tertiary lymph follicles (Magliozzi, et al., 2007). This type of inflammation, which is associated with the formation of subpial demyelinated lesions in the cerebral and cerebellar cortex is already present in early stages of MS, but gradually increases with disease duration (Lassmann, 2018).

Increasing evidence suggests that meningeal inflammation plays an important role in cortical GM pathology in MS (Magliozzi, et al., 2007) (Magliozzi, et al., 2010). Ectopic B cell follicle-like structures are detectable in the cerebral meninges of a large proportion of cases with SPMS (Pirko, et al., 2007) (Howell, et al., 2011) and those patients displayed a greater global meningeal inflammation that was associated with increased GM pathology. In this context, the presence of lymphoid aggregates represents a state of elevated meningeal inflammation that is likely to play a key role in the pathophysiology of cortical demyelination. In particular, microglial activation,

caused by the diffusion of inflammatory mediators from the meninges, could contribute to cortical pathology through the release of cytotoxic molecules (Howell, et al., 2011) with subsequent increase of demyelination. Interestingly, *in vitro* studies have also showed a toxic effect of the CSF of people with MS on cultured oligodendrocytes (Alcázar, et al., 2000) (Wentling, et al., 2019) leading to failure of remyelination through the induction of a proinflammatory environment which is hostile for repair (Larochelle, et al., 2021) (El Behi, et al., 2017).

Neuropathologists found that demyelination within the cortex is extensive, in particular in patients with a progressive disease course (Bø, et al., 2003) (Kultzelnigg, et al., 2005). In 20 brain samples of patients with MS (3 RRMS, 10 SPMS, and 7 PPMS), Bø and colleagues found an average of 26% of cortical area that was demyelinated. Demyelination in the cortex is mainly due to purely cortical lesions, with intracortical lesions accounting for 84% of cortical lesions and 86% of the cortical demyelinated area (Bø, et al., 2003). In general, cortical demyelination may lead to four different types of lesions described in histopathology: i) leukocortical lesions, which extend across both the WM and the GM (type 1), ii) intracortical lesions, found both in cerebellar and hemispheric cortical regions, which often develop around a blood vessel (type 2), iii) subpial lesions, which extend from the surface of the brain inward (type 3), and iv) lesions which extends throughout the full width of the cerebral cortex without crossing the edge with subcortical WM (type 4) (Bø, et al., 2003).

At the earliest stage of the disease, leukocortical lesions have been found to be the most frequent (50%), compared to subpial (34%) or intracortical (16%) lesions, and are often active, as reflected by the presence of myelin-laden macrophages (Lucchinetti, et al., 2011). In the study performed by Lucchinetti and colleagues, inflammatory infiltrates were observed in all lesion types by the presence of perivascular CD3+ T-cells and CD8+ T-cells, in respectively 82% and 77% of the studied lesions (Lucchinetti, et al., 2011). B-cells were observed in 27% of the cortical lesions and all the studied lesions contained microglial activation. By contrast, cortical lesions found at a later stage of the disease are most frequently subpial (Bø, et al., 2003) and represent a neuropathological hallmark of the disease (Junker, et al., 2020).

Lastly, similarly to what observed in the NAWM, diffuse microstructural modifications such as increased microglia activation, reduced axon densities (-33.0%compared to controls) and neuronal atrophy (13.6% smaller compared to controls) also affect the non-lesional normal appearing gray matter (NAGM) (Klaver, et al., 2015).

1.2.3 Neurodegeneration in MS underlies clinical progression

Clinical observations have repeatedly shown that the natural history of MS progression is usually independent of relapse activity with inflammation (Scalfari, et al., 2010) and they are inconsistent with the idea that inflammation is the sole power of accumulating disability and degeneration in people with MS. (Stys, et al., 2012) (Cree, et al., 2019).

Although the last two decades have seen an impressive progress in the understanding of the role of inflammation in the pathophysiology of MS, the processes underlying neuroaxonal degeneration, which is one of the major determinants of clinical disability and is present since the earliest stages of the disease, are still not completely understood (Tallantyre, et al., 2010).

In recent years, several pathways have been suggested to drive neurodegeneration in MS, including demyelination not followed by myelin repair, persistent inflammation, excitotoxicity, oxidative stress and ion channel dysfunction with energy dysfunction (Campbell, et al., 2014).

Among all these mechanisms, over recent years the failure of remyelination has progressively assumed an increasing role and is now considered as one of the major determinants of neurodegeneration and clinical progression in MS.

General mechanisms underlying myelin repair in MS

Endogenous myelin repair is a physiological response to a demyelinating event (Franklin & Goldman, 2015), which occurs in both the central (CNS) and peripheral nervous systems (PNS). In experimental models of toxin-induced demyelination, as well as in traumatic injuries, remyelination is normally extensive and complete (Lasiene, et al., 2008). Nevertheless, in MS, although myelin repair can be extensive in the early stages of the disease, it eventually fails resulting in chronically demyelinated lesions (Franklin & Goldman, 2015) (Patani, et al., 2007) (Patrikios, et al., 2006). A population of adult brain resident progenitor cells (oligodendrocyte progenitor cells or OPCs) are thought to mediate myelin repair by migrating into areas of demyelination and differentiating into actively myelinating oligodendrocytes (Zawadzka, et al., 2010). Over the past decades, OPCs have been extensively studied with the aim to elucidate the process of myelin repair for therapeutic exploitation.

Genetic lineage tracing methodologies have shown that NG2+ adult OPCs residing in the adult CNS are primarily responsible for remyelination following demyelinating insults (Tripathi, et al., 2010) (Zawadzka, et al., 2010). Oligodendrocytes surviving after a demyelination event may also contribute to remyelination by producing new sheaths (Jeffries, et al., 2016), even if they do not proliferate or migrate into the lesion (Crawford, et al., 2016). Moreover, the subventricular zone, one of the two neurogenic niches in the adult brain, is able to provide additional progenitor cells to the new myelinating cell population (Mecha, et al., 2013) (Xing, et al., 2014). Schwann cells,

which normally form PNS myelin, may sometimes contribute to myelin repair in the CNS, particularly in the spinal cord (Felts, et al., 2005).

Differently from other progenitor cells, OPCs persist into adulthood in the rodent and human brain, comprising 5–10 % of all CNS cells and a significant part of its glial cell population (Dawson, et al., 2000) (Duncan, et al., 2018). This cell population constitutes a stable pool of oligodendrocyte progenitors that undergo differentiation when needed, to remyelinate denuded axons under appropriate conditions (Miron, et al., 2011) (Werkman, et al., 2021).

In general, following specific stimuli, OPCs may undergo asymmetric division and migrate towards the lesion site, guided by chemoattractant factors such as Semaphorin 3F (Aigrot, et al., 2022) and at the lesion level, they start to differentiate into mature myelinating oligodendrocytes. A variety of factors regulate remyelination, including interaction of OPCs with cells of the neurovascular unit (Hamanaka et al., 2018) and necroptotic death of proinflammatory microglial cells, which is essential for the establishment of a non-inflammatory microenvironment that will permit myelin repair (Flyod, et al., 2019) (Galloway, et al., 2020).

Myelin secures saltatory conduction by affecting the distribution and spatial arrangement of a variety of voltage-dependent ion channel proteins along the node, paranodal and juxtaparanodal regions (Poliak & Peles, 2003) (Sherman & Brophy, 2005).Even though myelin sheaths of remyelinated axonal segments are thinner and shorter than those of non-demyelinated axons, they have the capacity to restore saltatory conduction (Franklin & Goldman, 2015) (Liebetanz & Merkler, 2006). Furthermore, restoration of impulse conduction resulting from myelin repair has been associated with relief from neurological symptoms. For instance, Bramow et colleagues demonstrated that the extent of remyelination in the spinal cord of progressive MS patients was inversely correlated with their level of disability (Bramow, et al., 2010).

Several lines of evidence indicate that axonal degeneration observed in chronically demyelinated lesions is the pathological correlate of permanent neurological dysfunction in MS patients (Alizadeh, et al., 2015) (Kornek, et al., 2000) (Trapp & Nave, 2008). Several transgenic models have shown that deletions of single myelin protein genes lead to secondary axonal degeneration and functional deficits (Griffiths, et al., 1998) (Lappe-Siefke , et al., 2003). Conversely, it has been shown that myelin repair is able to protect axons from degeneration (Irvine & Blakemore, 2008) (Kornek, et al., 2000) (Mei, et al., 2016). Protective effects of remyelination are exerted via multiple mechanisms, which include re-organization of nodal architecture (Coman, et al., 2006) (Black, et al., 2006), physical insulation of the denuded axon

from an unfavorable extracellular environment (Papadopoulos, et al., 2006), and recovery of metabolic support (Verden & Macklin, 2016).

Failure of myelin repair in MS

The process of spontaneous myelin repair in people with MS is globally inefficient. While several post-mortem studies have shown that the extent of remyelination is heterogeneous across patients with MS (Patrikios P, 2006) (Patani R, 2007), overall, less than 25% of MS brains are characterized by an extensive process of myelin repair in the white matter (Patrikios P, 2006). So far, only few neuropathological studies have investigated the process of remyelination occurring in the cortex of people with MS. These studies, in addition to confirming that even in cortical regions the extent of remyelination varies considerably across patients, have provided the notable evidence that myelin repair is significantly more extensive in cortical than in white matter lesions (Chang, et al., 2012).

Several factors have been shown to inhibit endogenous remyelination in MS, affecting OPC migration, maturation or function. Among the factors critically impacting the extent and the effectiveness of remyelination in each lesion, there are the different MS clinical subtypes, the duration of the disease, the degree of inflammation of the environment, the level of neuronal activity, the age-related cellular senescence, and the lesion location.

Luchetti and colleagues showed significant differences in remyelination efficiency between patients with a relapsing and those following a progressive course (Luchetti, et al., 2018) and it has been reported that patients in the PPMS group exhibit more extensive remyelination compared to those in the SPMS group (Bramow, et al., 2010), suggesting that remyelination efficiency may differ in different MS clinical subtypes, being most extensive in RRMS and least extensive in SPMS.

In chronic active MS plaques, typically known to either lack myelin repair or only be marginally remyelinated, the number of OPCs was lower compared to healthy controls, possibly due to the expression of Semaphorin 3A (Sema 3A), an OPC chemo-repellant (Boyd, et al., 2013). In contrast, active lesions containing a higher number of OPCs expressed low levels of Sema 3A mRNA overall and higher levels of Sema 3F mRNA, which is an OPC chemoattractant. Denuded axons have been shown to express a number of remyelination inhibitors, such as LINGO-1, a component of the Nogo receptor (Yin & Hu, 2014) and polysialic acid (PSA), an inhibitor of developmental myelination (Charles, et al., 2000). Axons and immune cells in chronic inactive lesions inhibit remyelination by expressing galectin-4, a factor that negatively regulates OPC differentiation (de Jong, et al., 2018) and lymphocytes derived from MS patients inhibit

remyelination in experimental models, through the secretion of CCL19 (El Behi, et al., 2017). Confirming the role of the immune system in regulating the process of myelin repair, Larochelle et colleagues recently showed that CNS-infiltrating T-helper17 cells form prolonged stable contact with oligodendrocytes and this contact worsens experimental demyelination, causes damage to human oligodendrocytes, and increases cell death (Larochelle, et al., 2021). In comparison to T-helper2 cells, both human and murine T-helper17 cells express higher levels of the integrin CD29, which is linked to glutamate release and can induce cell stress and consequently cause a decrease in myelination (Larochelle, et al., 2021).

Several lines of evidence indicate that also neuronal electrical activity plays a key role in promoting remyelination. Ronzano and colleagues demonstrated that the interaction between microglia and Ranvier's nodes depends on the neural-activity though a potassium mediated mechanism (Ronzano, et al., 2021). This interaction is essential to promote the microglial switch towards a pro-myelinating phenotype which results in an increased remyelination, corroborating the hypothesis of neuronal-dependent process of remyelination (Mitew, et al., 2018) (Ortiz, et al., 2019)

The pattern of chronically demyelinated MS lesions either being devoid of OPCs or containing OPCs that fail to differentiate and remyelinate suggests that ageing and the processes that drive ageing may be a limiting factor for remyelination in advanced stages of multiple sclerosis (Boyd, et al., 2013) (Chang, et al., 2002).Interestingly though, endogenous l remyelination at the cortical level occurs in most patients with MS regardless of chronological age or disease duration (Chang, et al., 2012).

Ageing has been shown to affect myelin repair in animal models of demyelination (Cantuti-Castelvetri, et al., 2018) (Hampton, et al., 2012). In aged mice, in the cuprizone model of demyelination, remyelination failure is associated with inefficient recruitment of histone deacetylase, which allows accumulation of transcriptional inhibitors, preventing the subsequent surge in myelin gene expression (Shen, et al., 2008).OPCs from aged rats exhibit features of cellular senescence with increased levels of DNA damage and mitochondrial dysfunction (Neumann, et al., 2019).

Nevertheless, OPC senescence is not only associated with chronological ageing. There is in vivo evidence of other triggers of senescence in OPCs such as the exposure to Aβ oligomers which elicited cellular senescence in murine OPCs and inhibited myelin sheet (Horiuchi, et al., 2012). OPCs expressing upregulated p16, p21 and SA-β-Gal have been identified in association with Aβ plaques in Alzheimer's disease (AD) mouse models and human AD brains. Senolytic

treatment to remove senescent OPCs in the APP/PS1 model of AD attenuated neuroinflammation and cognitive deficits, indicating that OPC SASP contributes to neuroinflammation and functional impairment (Zhang, et al., 2019). It is plausible that the accumulation of senescent OPCs in MS could contribute to sustaining neuroinflammation in chronic demyelinated lesions in a similar manner.

Cellular senescence has also been shown to affect neurons, microglia, astrocytes, pericytes and endothelial cells (Bhat, et al., 2012) (Yamazaki, et al., 2016) (Safaiyan, et al., 2016); which may all directly or indirectly influence the efficiency of myelin repair. For instance, neurons with a senescent phenotype have been shown in cortical MS demyelinated lesions. GL13 histochemistry of subpial demyelinated cortical lesions and normal appearing cortex revealed granular lipofuscin deposits in numerous neurons, indicating that neurons in SPMS exhibit a senescence-like phenotype (Kritsilis, et al., 2018). Nevertheless, it is not known whether the axons of neurons exhibiting a senescence phenotype are receptive to remyelination. Recently, astrocytes aged in culture were shown to exhibit a senescent phenotype associated with the secretion of extracellular vesicles (EVs) that unlike EVs from young astrocytes did not sufficiently support for oligodendrocyte differentiation suggesting that astrocytic senescence may be detrimental for OPC differentiation and myelin repair (Willis, et al., 2020).

Epithelial cell and pericyte senescent phenotype are accompanied by reduced tight junction protein coverage and impaired blood-brain barrier (BBB) integrity. This has been proposed by Yamazaki and colleagues (Yamazaki, et al., 2016) as a factor contributing to BBB augmented permeability during ageing, leading to the entry of neurotoxic and neuroinflammatory agents from the periphery to the CNS parenchyma. Fibrinogen, a blood-derived coagulation factor entering the brain though the disrupted BBB, has been found to interfere with OPC differentiation into mature oligodendrocytes, and to inhibit remyelination of lysolecithin-induced demyelinated lesions (Petersen, et al., 2017). Interestingly, an increase in the amount of fibrinogen in the CSF was also associates with a greater cortical lesion load in the early stages of MS and was associated with greater levels of soluble CD163 (Magliozzi, et al., 2019). This has been proposed as a potential predictor of MS activity (Stilund, et al., 2015) as it was found to be expressed on macrophages and microglia in MS plaques (Zhang, et al., 2011).

Finally, a relevant factor which has to be considered when exploring the myelin repair efficacy is the lesion location. The evidence that periventricular lesions exhibit less extensive remyelination in comparison to subcortical ones (Patrikios, et al., 2006), or that unlike in other brain regions cerebellar lesions remain largely demyelinated (Goldschmidt, et al., 2009), highlights the

relevance of lesion localization in explaining the across-lesion heterogeneity of the extent of remyelination. Of great importance is the notion that OPCs have been demonstrated to be more numerous in cortical MS lesions, which consequently are more frequently remyelinated than white matter lesions (Albert, et al., 2007) (Strijbis, et al., 2017) regardless of disease duration or chronological age of the patient (Chang, et al., 2012)

Chang and colleagues showed that twenty-eight of 57 (49%) chronic leukocortical lesions had evidence of remyelination in the cortical portion and variable amounts of remyelination in the white-matter portion. Overall, the extent of remyelination in gray matter exceeded that observed in the white matter in 24 of the 28 leukocortical lesions with remyelination in gray matter (Figure 3) and this could be due to the fact that the density of oligodendrocytes in the gray-matter portion of leukocortical lesions was increased 6.8 fold compared to the white-matter portion ($37.3/mm^2$ vs. $5.5/mm^2$, $P<0.0001$) (Figure 3)

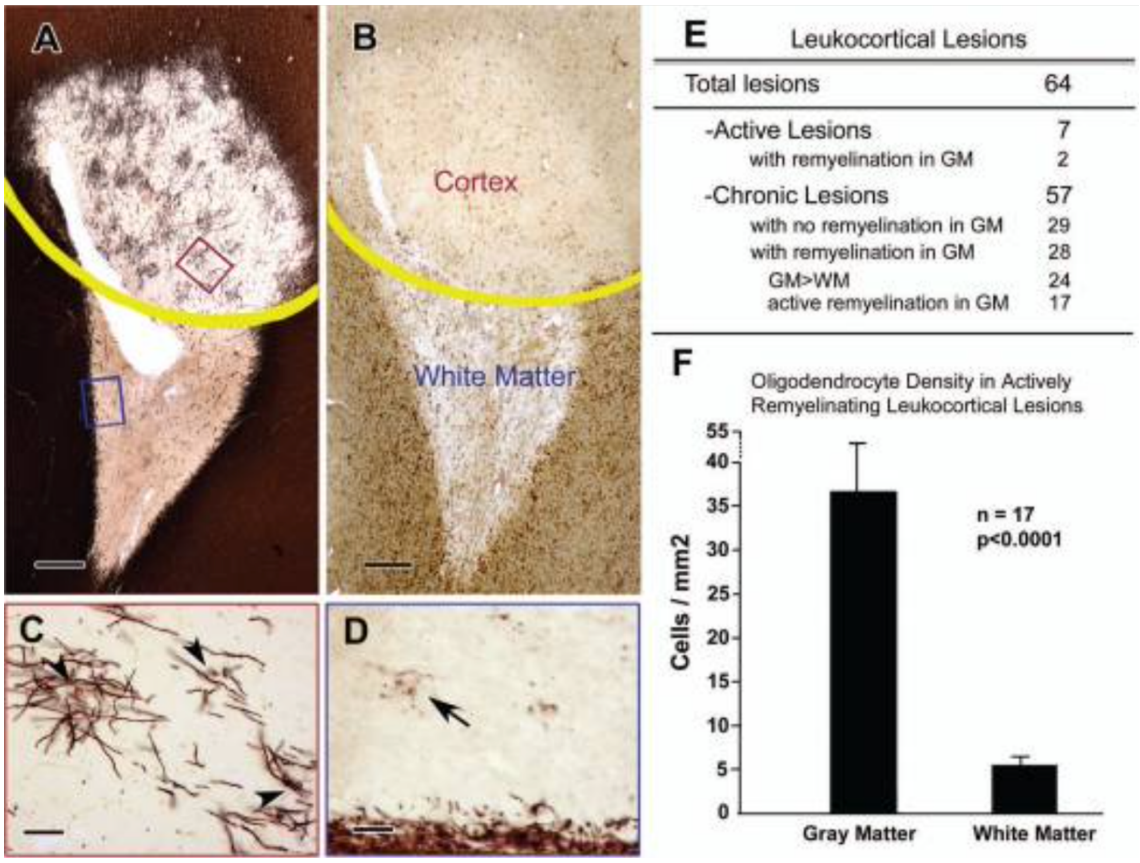


Figure 3. From Chang et al 2012. Remyelination in Chronic Leukocortical Lesions
PLP immunohistochemistry of a leukocortical lesion shows significant remyelination in the demyelinated cortex compared to the demyelinated white matter (Panel A, yellow line shows the boundary between cortex and white matter). MHC Class II staining (Panel B) identifies this lesion as chronic. The PLP-positive cells in demyelinated cortex have features of actively-remyelinating oligodendrocytes (Panel C is high magnification of the red box in Panel A), whereas those in demyelinated white matter often extend dystrophic processes with no apparent connection to myelin internodes (Panel D is high magnification of the blue box in Panel A). Twenty-eight chronic leukocortical lesions (49%) show evidence of remyelination (Panel E). Of these, 24 showed a greater extent of remyelination in the gray matter (GM) compared to that in the white matter (WM), and 17 of these 24 (70%) show evidence of active remyelination. The density of oligodendrocytes in the gray-matter portion of the actively-remyelinating lesions greatly exceeds the oligodendrocyte density in the white-matter portion (Panel F, $P < 0.0001$). The scale bars in Panels A and B represent 400 μm; the scale bars in Panels C and D represent 50 μm; the error bars in Panel F represent standard error of mean

Chapter 1.3 – Imaging myelin loss and repair in MS: what we know and what we need to know

The interest of measuring in vivo the processes of myelin loss and repair in MS, as well as the effects of promyelinating compounds in patients with this disease has fostered the need for novel imaging metrics being both sensitive and specific to myelin content. Indeed, conventional T2-w MRI is very sensitive in identifying WM lesions in MS, but they are not specific for the pathological substrates underlying lesional pathology. To overcome this problem, several imaging techniques have been developed to measure myelin content changes in vivo, based on the use of positron emission tomography and quantitative MRI.

1.3.1 PET-based metrics to image myelin content changes in MS

Positron emission tomography (PET)-based strategies with myelin-binding tracers have been proposed to investigate in vivo myelin content change in MS.

One of the first radiotracers to be identified is a stilbene derivative called BMB (1,4-bis(p-aminostyryl)-2-methoby benzene) (Stankoff, et al., 2006), which paved the way for the investigation of several other stilbene-derived compounds such as BDB, CIC (Wang, et al., 2009), GE3111 (Cotero, et al., 2012), and [11C]-MeDAS (Wu, et al., 2010) (de Paula Faria, et al., 2014). The ability of these tracers, originally developed as amyloid markers, in identifying myelin is probably due to a shared beta-sheet structural conformation within amyloid plaques and the myelin sheath (Bajaj, et al., 2013). In line with this hypothesis, other amyloid markers of the benzothiazole family were investigated for their potential to be used as myelin markers. In particular, the thioflavin T derivative 2-(4'-methylaminophenyl)-6-hydroxybenzothiazole (Pittsburg Compound B, PiB) was shown to stain myelin ex vivo in rodent and human post-mortem brain samples (Stankoff, et al., 2011). Interestingly, [^{11}]C-PiB uptake was initially decreased following a lysolecitine injection in a rat model of disease, and progressively recovered in parallel with dynamic remyelination (de Paula Faria, et al., 2021).
Other promising myelin radiotracers have also been assessed, including the 18F-PENDAS111, and 1,2,3-triazole derivative TAFDAS (Wu, et al., 2017), as well as coumarine-based molecular probes (Wang, et al., 2011). In an in vivo PET study on baboons comparing some stilbene and benzothiazole compounds, it has been shown that the second-generation fluorinated tracers

18F-florbetaben and 18F-florbetapir exhibit a higher signal to noise ratio in the white matter than 11C-PiB or 11C-MeDAS (Auvity, et al., 2020).

Myelin PET studies using 18F-florbetaben in patients with all MS forms showed a lower tracer uptake inside and outside visible lesions, correlating with cognitive decline and increased WM lesion volume over time (Pytel, et al., 2020). Myelin PET also demonstrated a high heterogeneity across lesions with respect to the extent of demyelination (Fisher, et al., 2017) (Bodini, et al., 2016).

A key application of PET with myelin radiotracers is in the longitudinal analysis of myelin content changes over time, thus offering the possibility to explore in vivo the dynamics of remyelination (Bodini, et al., 2021). A longitudinal study published by our group in 2016 has shown the ability of [^{11}C]-PiB with a high-resolution research camera to capture demyelination and remyelination in single WM lesions over time (Bodini, et al., 2016). To do so, first selected a reference region using a supervised clustered method and by means of the Logan graphical analysis we extracted the distribution volume ratio (DVR) of [^{11}C]-PiB (Veronese, et al., 2015). Then, using a voxel-wise approach, dynamic indices of global myelin content change, as well as of demyelination and remyelination were calculated for each patient. This approach allowed to demonstrate a significant across-patient heterogeneity in the individual WM remyelination potential, which appeared to be inversely correlated with clinical disability (Figure 4).

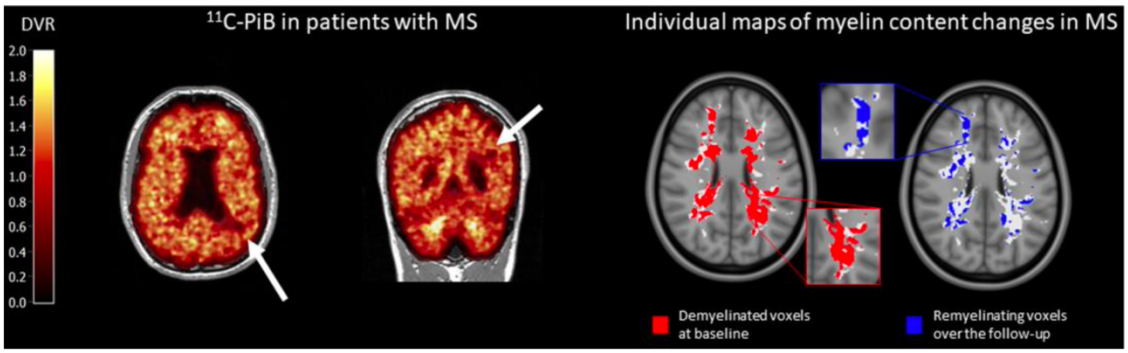


Figure 4. Targeting demyelination and remyelination in MS. 11C-PiB DVR map of a patient with multiple sclerosis overlaid onto the corresponding T1-weighted MPRAGE scan. Individual maps of myelin content changes obtained after thresholding longitudinal 11C-PiB DVR maps of the same patient with multiple sclerosis, overlaid onto a template MRI scan in standard space. From Bodini B, Tonietto M, Airas L, and Stankoff B (2021). Positron Emission Tomography in Multiple Sclerosis: Straight to the Target. Nature Rev Neurol. 2021 Nov;17(11):663-675

However, the main disadvantage of [11C]-labeled compounds is the short half-life of just 20 minutes. The use of fluorinated amyloid tracers, with a longer half-life of 110 min, could help overcome this issue and represent offering promising alternatives for myelin PET imaging (Auvity, et al., 2020) (Pietroboni, et al., 2019) (Zeydan, et al., 2018). Using a fluorinated

compound ([18F]-florbetapir), Pietroboni and colleagues investigated damaged and normal-appearing WM in patients with MS and evaluated correlations between CSF β-amyloid 1-42 (A β) levels, [18F]-florbetapir uptake and brain volumes (Pietroboni, et al., 2019). Reduction of tracer uptake was found in damaged WM compared to the normal appearing WM. NAWM uptake appeared to be lower in active versus non-active patients, with the CSF A β concentration being a predictor of both NAWM standard uptake value (SUV) and NAWM volume only in active patients. These findings may suggest that PET signal in the NAWM is at least partially influenced by amyloid trafficking between the brain and the CSF (Pietroboni, et al., 2019).

In general, some considerations have been made about the use of PET for the measure of myelin in vivo. First, the high cost and reduced availability of PET scanners limit the possible application of this technique on a large scale, rather favoring its use as an intermediate tool towards the development and validation of more accessible multiparametric MRI-based techniques for myelin quantification. Other important issues are the spatial resolution of PET and the partial volume effect that must be considered in data interpretation. Moreover, when myelin dynamics are explored in longitudinal datasets, the reproducibility of quantitative methods used to estimate demyelination/remyelination has to be assured, often relying on the application of dynamic acquisitions with quantitative approaches over semi-quantitative ones, and on the collection of test–retest data in healthy controls (Bodini, et al., 2021). In addition, the contribution of non-myelin proteins to the white matter and thalamic signal obtained with some repurposed amyloid tracers cannot be completely ruled out (Pietroboni, et al., 2019). Lastly, the only non-invasive validated quantification of myelin tracers available so far for the white matter is based on the automatic extraction of a reference region from the grey matter (Veronese, et al., 2015), which challenges its application for the measure of myelin in cortical and deep grey matter regions.

1.3.2 Quantitative MRI to image myelin content changes in MS

Conventional MRI is insensitive to the heterogeneity of focal multiple sclerosis lesions and to the pathology affecting CNS tissue outside multiple sclerosis lesions (normal-appearing white and grey matter) and is unable to depict the level of damage within different CNS tissue components, such as myelin, axons, and glia (Granziera, et al., 2021).

Quantitative MRI can potentially address these needs by providing more sensitive measures of multiple sclerosis pathology and more specific information regarding which tissue component has been damaged (Figure 5).

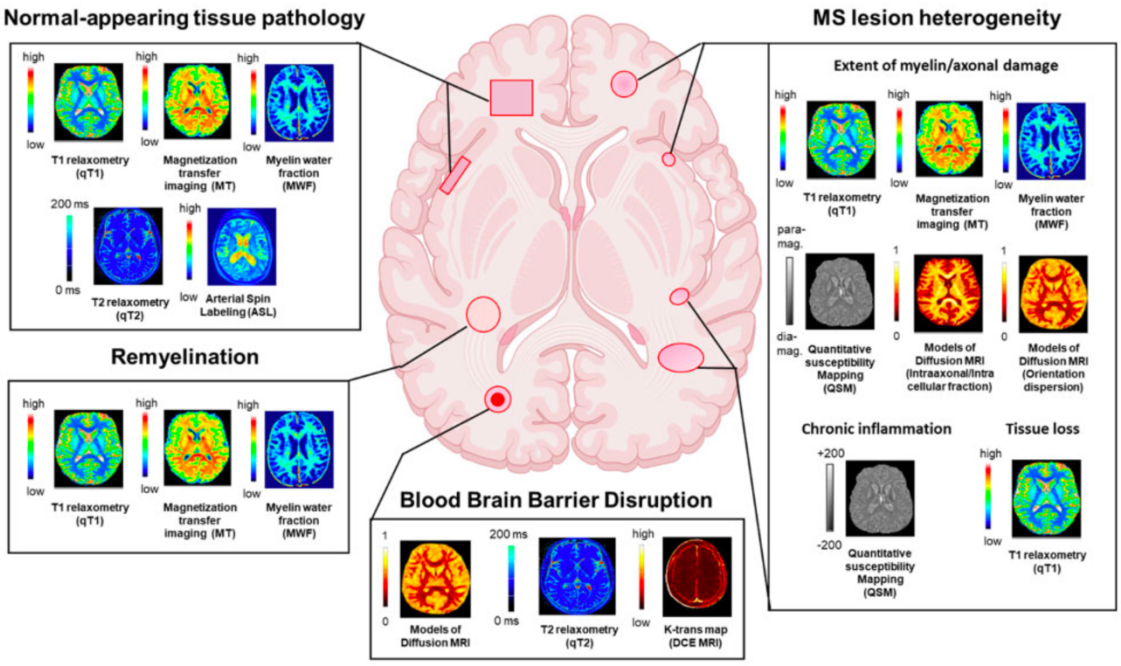


Figure 5. From Granziera et al., 2021

In the evaluation of myelin content and myelin content change, different quantitative MRI techniques has been proposed: among all, for myelin water fraction (MWF), magnetization transfer ratio (MTR), inhomogeneous magnetization transfer ratio (ihMT), quantitative magnetization transfer (qMT), quantitative susceptibility mapping (QSM), g-ratio, Ultrashort echo time (UTE), T1/ T2 weighted MRI and R2*, a histologic validation in animals and/or human studies has been provided.

A recent review by van der Weijden and colleagues found that overall, the correspondence of all MRI methods combined with myelin histology in animals is R2=0.54 (SD=0.30, n=446). Forest plot analysis of individual MRI methods shows that ihMTR has the highest correspondence with myelin histology (R2=0.94, n=3, N=1), followed by QSM (R2=0.85, n=29, N=2), g-ratio (R2=0.69, n=12, N=1), quantitative qMT (R2=0.60, n=124, N=10), myelin water fraction MWF (R2=0.55, n=73, N=5), T1 mapping (R2=0.55, n=15, N=2), UTE (R2=0.51, n=15, N=1), MTR (R2=0.42, n=145, N=10), and T2 mapping (R2=0.37, n=30, N=3).

On the other hand, the correspondence of the combined ex-vivo human myelin MRI methods with histology is R2=0.54 (SD=0.18, n=340). In this case, forest plot analysis of individual MRI methods showed that the highest MRI-histological correspondence was found for MWF (R2=0.68, n=28, N=2), followed by MTR (R2=0.65, n=98, N=6), qMT (R2=0.60, n=52, N=2), T1 mapping (R2=0.48, n=91, N=6), T2 mapping (R2=0.45, n=34, N=4), R2* (R2 =0.18, n=14,

N=2), and QSM (R2 =0.07, n=11, N=2) (van der Weijden, et al., 2021). These results are in line with the recently published meta-analysis by Mancini and colleagues (Mancini, et al., 2020), which showed that the largest studies exploring the association between MRI metrics and myelin content, indicate that MT and T2 relaxometry (i.e MWF) correlate better than any other sequence with myelin content (Figure 6) .

Figure 6. From Mancini et al 2020. Bubble chart of R2 values between a given MRI measure and histology for each study across MRI measures, with the area proportional to the number of samples. To see the interactive figure: https://neurolibre.github.io/myelin-meta-analysis/02/closer_look.html#figure-3

It is worth noting that the available data derived from animal models, ex-vivo studies on human brains, and in-vivo repeatability studies is still limited for most MRI methods, thus precluding a definite conclusion on the most optimal MRI method for myelin quantification. Moreover, an accurate comparison of these studies is challenged by significant differences in the employed methodologies. For instance, differences in sample preparation, especially in the use of fixation, was suggested as a negative influencer of the MRI correspondence with myelin histology (Schmierer, et al., 2008). Nevertheless, van der Weijden and colleagues found only a trend towards an effect of sample preparation on the correlation between MRI and histology in animal studies (in-vivo vs. ex-vivo MRI) (van der Weijden, et al., 2021). This trend was not found in human studies (fresh vs. fixated samples). It has been suggested that the fixation process interacts with relevant macromolecules, thereby altering their physical characteristics and thus magnetization transfer between these macro- molecules and the free water pool (Schmierer, et al., 2010). However, the fact that similar results were obtained when fresh and fixated human

samples were used, indicates that the effect of sample preparation in animal studies is probably not caused by the fixation process itself, but is likely due to a difference in acquisition of the imaging signal between in-vivo and ex-vivo samples, such as distortion of magnetic field homogeneity (van der Weijden, et al., 2021).

The distinction of different water pools, including the myelin water pool, in the CNS may be achieved by using both MTR and MWF (Granziera, et al., 2021). Nevertheless, MTR, as well as MWF, is also sensitive to the relative presence of extracellular water and their values may be influenced by the presence of oedema and or inflammation (Vavasour, et al., 2012) (Bonnier, et al., 2014). Consequently, these myelin-related measures exhibit different specificity regarding myelin content and myelin-related pathology in multiple sclerosis.

MWF shows strong correlations with myelin staining in both lesions and normal-appearing tissue in histological specimens of human brain (on average in lesions and normal-appearing tissue: $r2 = 0.67$, $p<0.0001$) (Laule, et al., 2006) (Laule & Wayne Moore, 2018). Whether MWF is also sensitive to accumulation of extracellular iron remains to be demonstrated. A recent post-mortem study attempted to answer this question by imaging brain specimens with two different techniques measuring MWF before and after a de-ironing procedure (Birkl, et al., 2019). This work concluded that both were sensitive to brain iron content, but the application of de-ironing procedures might have affected the iron content within myelin and, as a consequence, altered myelin properties.

Vargas and colleagues found that MWF increased over 6 months following the appearance of new enhancing lesions, hypothetically reflecting a repairing process (Vargas, et al., 2015). Moreover, MWF moderately decreased over time (–8%) in the normal-appearing white matter of a small group of patients and was found to be abnormal in the brain and cervical spinal cord of patients with PPMS compared to controls (Kolind, et al., 2015). MWF was also found to be significantly correlated with clinical disability (Laule, et al., 2010).

These data, together with its good specificity for myelin, suggest that MWF might be a good tool for the *in vivo* evaluation of WM and cortical GM myelin content as suggested by Rahmanzadeh and colleagues (Rahmanzadeh, et al., 2021).

MTR has been validated by post-mortem studies and shows high correlations with myelin ($r = -0.84$, $p<0.001$) and axon density ($r = -0.80$, $p< 0.0001$) (Schmierer, et al., 2004) specifically in WM (van Waesberghe, et al., 1999). MTR signal has also been found to significantly correlate with the degree of cortical demyelination (Chen, et al., 2013). Nevertheless, a recent MRI-pathology study has shown that MTR sampled in WM lesion and WM non-lesional tissue in

multiple sclerosis brains is associated not only with myelin density but also with the number of astrocytes, damaged mitochondria and microglia cells (Moccia, et al., 2020). Interestingly, the same study found that the MTR specificity for myelin varies across different brain regions. Despite the sensitivity for myelin is relatively high and homogeneous across the brain, its specificity for myelin seems to be higher in cortical GM regions compared to WM areas. In particular, looking at the differences in the associations of MTR and immunostaining intensities between different brain regions and using NAWM as reference, the association between MTR and macrophage density (CD68) and with and astrocytic density (GFAP) was stronger in WM lesions compared to cortical GM lesions (Moccia, et al., 2020). The notion of a relative minor inflammation and astrogliosis affecting cortical GM in comparison with WM (Peterson, et al., 2001) (Chang, et al., 2012), may account, at least to some extent, for the higher specificity of MTR for myelin in cortical regions.

From an *in vivo* point of view, MTR changes in normal-appearing white matter preceded the appearance of gadolinium-enhancing lesions in patients with MS (Filippi, et al., 1998) and recovered following the acute phase, especially in treated patients (Bown, et al., 2020). For lesions that changed from hypointense to isointense on T1-weighted images, MTR increased significantly during 6 months of follow-up (van Waesberghe, et al., 1998) and showed longitudinal changes consistent with demyelination and remyelination in different regions of active lesions in the 3 years following treatment with DMTs (Chen, et al., 2008).
Interestingly, MTR also appeared to be lower in the outer compared to the inner cortical layers in the brain (Samson, et al., 2014) and in the subpial region compared to the central region in the spinal cord (Kearney, et al., 2014). These findings may be consistent with more severe levels of demyelination in regions close to the CSF, but may also, at least in part, be explained by partial volume effects.

Chapter 2 – Rational and aims of this PhD work

Although our knowledge concerning the biologic process of myelin loss and repair has progressively increased over time, allowing us to assert that multiple sclerosis is a neurodegenerative disorder characterized by immune-mediated myelin loss and a concomitant failure of physiological myelin repair processes, to date the clinical impact of myelin repair, particularly at the cortical level, remains largely unknown.

So far, only few neuropathological studies have investigated the process of remyelination occurring in the cortex of people with MS. As mentioned above, these studies clearly demonstrated that myelin repair is significantly more extensive in cortical than in white matter lesions (Chang, et al., 2012). Moreover, these data indicate that cortical remyelination occurs in most patients with MS regardless of chronological age or disease duration (Chang, et al., 2012). Taken together, the results of these neuropathological studies suggest that cortical areas might represent an excellent context for exploring endogenous myelin regenerative responses in MS and may represent a more accessible therapeutic target for promyelinating therapies compared to white matter lesions. Therefore, the quantification of cortical remyelination with imaging techniques as a primary outcome in myelin repair clinical trials could dramatically increase the chance of identifying a successful myelin repair therapy for people with MS.

So far, the in-vivo measure of myelin content changes at the cortical level in patients with multiple sclerosis has proven to be challenging. This is largely due to the lack of appropriate imaging methodologies to measure patient-specific profiles of myelin content changes in the cortex of people with MS, which is the essential step to explore these processes in large cohorts of patients with all forms of this disease. As a result, key information on the clinical relevance of cortical myelin loss and repair, and on the main factors influencing these processes over the course of the disease remain unknown.

Against this background, this thesis focuses on addressing the following two questions:

1)Is the in-vivo imaging identification of individual profiles of cortical myelin loss and repair in patients with MS possible

2) Is the process of cortical remyelination in MS effective in preventing the progression of cortical atrophy and clinical disability over time?

Chapter 3 - Results

In the first part of this Chapter, I will address the first question of my research project, presenting the method I have developed and validated during my PhD to generate patient-specific MTR-based maps of cortical demyelination and remyelination. This novel approach has been tested and applied in a population of 15 Highly active RRMS patients and 7 healthy controls.

In the second part of this Chapter, I will address the second question of my research project, presenting the results of a multi-center study conducted on 140 patients with all forms of MS followed-up for 5 years in three centers of the MAGNIMS (Magnetic Resonance Imaging in MS) consortium. In particular, generating MTR-based maps of cortical myelin content changes in this large population of patients, I described for the first time cortical myelin loss and repair in all clinical forms of MS, identified all factors significantly affecting these processes and explored the predictive value of cortical demyelination and remyelination on the development of cortical atrophy at 1 year and on the accumulation of clinical disability at 5 years.

Chapter 3.1 - A novel clinically relevant method to measure patient-specific maps of cortical demyelination and remyelination in patients with multiple sclerosis

Introduction:

Cortical demyelination, particularly in subpial regions, is considered a specific neuropathological hallmark of multiple sclerosis (MS) (Junker, et al., 2020). Post-mortem data have shown that the process of myelin repair could be more efficient in cortical areas, suggesting that cortical regions may be an excellent context for exploring endogenous myelin regenerative responses in MS, which are thought to play a key neuroprotective role on demyelinated axons (Albert, et al., 2007) (Chang, et al., 2012) (Strijbis, et al., 2017). Indeed, the use of imaging metrics reflecting cortical remyelination as primary outcome measures in clinical trials has been proposed as a means of increasing the chance to identify successful therapies for myelin repair in MS (Chang, et al., 2012).

Magnetization transfer ratio (MTR), an imaging metric sensitive to myelin content changes in MS (Schmierer, et al., 2004) has been shown to be lower in cortical lesions than in normally myelinated cortex (Chen, et al., 2013) and has been successfully employed to generate maps of myelin loss in cortical grey matter (Derakhshan, et al., 2014). It is worth noting that processes other than myelin content changes, such as axonal loss, oedema and inflammation, may affect the MTR signal in MS (Schmierer, et al., 2004). This is particularly true for white matter (WM) regions, where up to 30% of the MTR signal can be explained by oedema and inflammation (WM), thus limiting the specificity of this technique for myelin in this brain region (Peterson, et al., 2001). However, the evidence that the extent of oedema and inflammation is less pronounced in grey matter regions compared to WM areas together with the recently introduced notion of a relatively higher MTR specificity for myelin in cortical grey matter areas (Moccia, et al., 2020) support the application of this technique to explore myelin loss and repair in cortical regions. Since the use of MTR in the white matter is limited by the suboptimal specificity of this technique for myelin in this particular brain region (Moccia, et al., 2020), other imaging techniques have been proposed to measure myelin content changes in white matter lesions of patients with MS (Bodini, et al., 2016). In particular, positron emission tomography with myelin-targeting

radiolabeled compounds offers the highest possible specificity for myelin in the white matter and been already successfully employed to quantify myelin content changes in WM lesions in MS (Bodini, et al., 2021). Using PET with Pittsburgh compound B ([11C]PiB), an amyloid tracer which has shown to bind selectively to myelin structure in the brain (Stankoff, et al., 2011) (Bajaj, et al., 2013), we previously showed that spontaneous remyelination is heterogeneous and clinically relevant in MS (Bodini, et al., 2016).

The objective of this study is to generate for the first time MTI-based metrics reflecting myelin loss and repair at the cortical level and to combine them with [11C]PiB-PET-derived parameters to explore the clinical relevance of individual profiles of cortical and white matter lesion myelin content changes in patients with MS.

Methods:

Subjects and study design:

Imaging and clinical data were retrospectively collected from 20 patients with acutely active relapsing-remitting MS according to the revised McDonald criteria14 (women=10; age 30.9 ± 4.6), and from 8 healthy controls with no history or signs of neurological and psychiatric diseases (women=7; age: 31.4 ± 6.5) were enrolled at the Centre d'Investigation Clinique (CIC) Neurosciences of the Pitié-Salpêtrière Hospital in Paris, France from 2009 to 2013. Main inclusion criteria were: i) age≥18 years; ii) >1 month after corticosteroid or immunosuppressive therapy; iii) presence of at least one gadolinium-enhancing MRI lesion at baseline or within the previous three months to identify patients with active disease.

Exclusion criteria were: i) impossibility to perform a PET and MRI exam for any reason; ii) severe or uncontrolled renal, hepatic, hematological, gastrointestinal pulmonary or cardiac disease; iii) other chronic neurological disease; iv) prior participation in other research protocols or clinical investigations in the previous year that would result in a radiation exposure exceeding the annual guidelines; v) for women: pregnancy, lactation, lack of efficient contraception. Patients underwent a full neurological examination and were scored on the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) and on the Multiple Sclerosis Severity Scale (MSSS) (Roxburgh et al., 2005).

All participants underwent MRI and dynamic [11C]PiB-PET at study entry. All patients except one, who left the study due to personal reasons after the baseline imaging assessment, repeated the imaging protocol after 2.5 months on average10 (mean 2.46, range 1-4). This short follow-up was chosen based on previous in-vivo data on lesional myelin repair, suggesting that a time-frame between 2 and 4 months following the appearance of a new lesion maximizes the

probability to capture changes in myelin content (Chen, et al., 2008). Due to the suboptimal quality of 5 MT scans (1 patient scan and 1 healthy control scan at the first-time point and 3 patient scans at the second time-point) owing to movement artefacts, our cross-sectional analysis was performed on 19 patients with MS and 7 healthy controls while our longitudinal analysis was focused on 15 patients and 7 healthy controls who had a complete set of good quality images at both time-points. All participants provided written informed consent and the study was approved by the local ethics committee.

Image acquisition

MRI was performed using a Siemens TRIO 3T MRI scanner, and included the following sequences: 3D-T1-weighted magnetization prepared rapid gradient echo (T1-w MPRAGE, TR/TE: 2300/2.98 ms, inversion time: 900 ms, resolution: 1.0x1.0x1.1 mm3); pre- and post-gadolinium T1 spin-echo (TR/TE: 700/14 ms, resolution: 1.0x1.0x3.0mm3); T2-weighted image (T2-w, TR/TE: 4100/83 ms, resolution: 0.9x0.9x3.0 mm3); (iii) 3D fluid-attenuated inversion recovery (FLAIR, TR/TE: 8880/129 ms, inversion time: 2500 ms resolution: 0.9x0.9x3.0 mm3); and 3D gradient-echo with (Mton) and without (Mtoff) magnetization transfer (MTI, TR/TE: 35/5 ms resolution: 1.0x1.0x2.0mm3).

PET examinations were performed on a high-resolution research tomograph (HRRT; CPS Innovations, Knoxville, TN), which achieves an intra-slice spatial resolution of ~2.5mm full width at half maximum. The 90-minute emission scan was initiated with a 1-minute intravenous bolus injection of [11C]PiB (injected activity = 358 ± 34 MBq)18.

Image post-processing

Native space:

In all subjects, MTR maps were generated using the equation MTR=(Mtoff-Mton)/Mtoff.

[11C]PiB-PET dynamic data were corrected for motion using a frame-by-frame realignment algorithm based on SPM (v8.0, https://www.fil.ion.ucl.ac.uk/spm/). Voxel wise distribution volume ratio (DVR) maps were obtained with Logan graphical analysis based on reference region extracted from the grey matter using a supervised clustering algorithm (Veronese, et al., 2015).

All images at each time-point, including MTR and DVR maps, were rigidly aligned to the corresponding T1-w scans using FLIRT (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/). Derived transforms were used to align WM lesion masks, contoured on T2-w images with reference to the corresponding FLAIR sequence, to T1-w scans. After performing a “lesion-filling” procedure in

patients (Chard et al., 2010). Cortical GM, WM and cerebrospinal fluid (CSF) were segmented in all subjects on T1-w scans using Freesurfer (https://surfer.nmr.mgh.harvard.edu). In each subject, a cortical-relative distance map between the CSF and the WM [distance from CSF/(distance from CSF + distance from WM) and a WM distance map from ventricles were generated using FSL (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/).

Standard space:

In each patient, the 3D-T1 MPRAGE images acquired at the two time-points were aligned to each other to create a "halfway space using Freesurfer". This halfway image was then normalized to the MNI152 standard space using a non-linear registration with ANTs (http://stnava.github.io/ANTs). Derived transformations were then combined to move all images (MTR and DVR maps, as well as segmented regions) from native space to MNI, passing through the halfway space in patients.

MTR-derived indices of cortical myelin content changes

Patient-specific maps of cortical demyelination were generated at both time-points and used to compute MTR-derived individual indices of cortical remyelination and demyelination, through the following steps:

(i) Regions of significant difference in cortical MTR between patients and healthy controls were identified using a voxel-wise permutation-based two-sample t-test adjusted for age and sex, thresholded at $p<0.05$, after threshold-free cluster enhancement correction (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/Randomise/UserGuide). In these significant regions, the MTR mean relative difference between patients and healthy controls was computed [(mean MTR in healthy controls – mean MTR in patients)/mean MTR in healthy controls] and employed as threshold to classify cortical voxels as "demyelinated" or "normally myelinated" in patients.

(ii) We then calculated the relative difference between the MTR value of any given cortical voxel in patients and the average MTR value of all voxels in healthy controls localized at the same distance than the given voxel from the external CSF (thus suffering from the same degree of partial volume effect). If this difference was greater than the previously calculated threshold, that cortical voxel was classified as "demyelinated", otherwise as "normally myelinated". As a result, we generated a map of cortical demyelination for each patient at each time-point. (Figure 7).

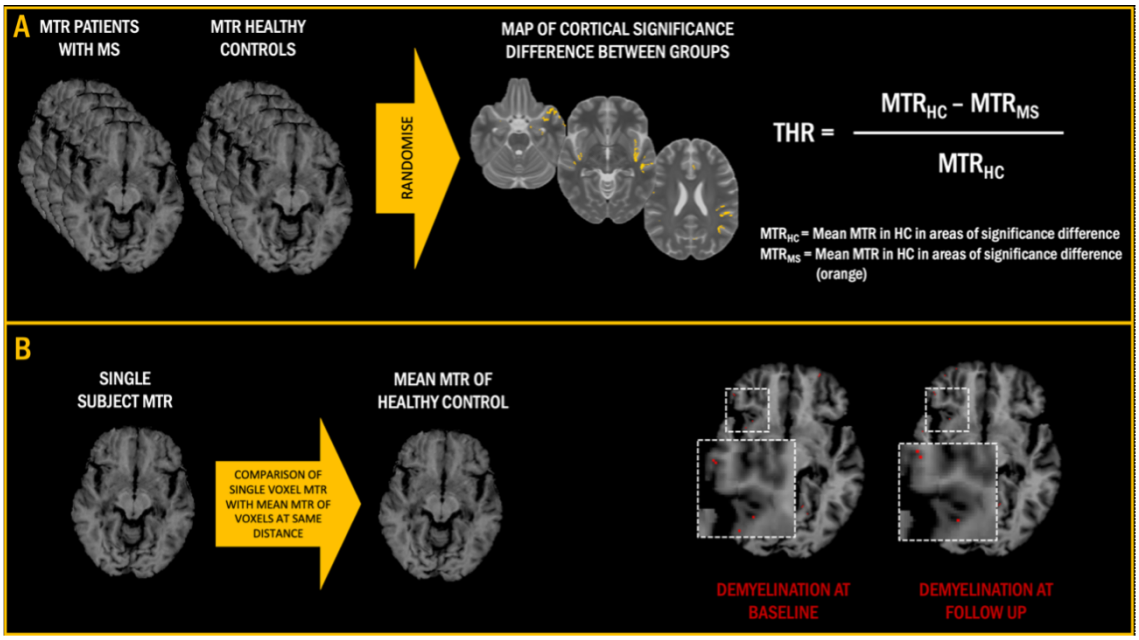


Figure 7: Flowchart of the image analysis pipeline applied to generate individual maps of cortical demyelination, based on MTR. (A) Definition of a threshold to identify cortical demyelinated voxels in patients compared to healthy controls. Regions of significant group differences between MTR maps of patients with MS and healthy controls were identified through voxelwise nonparametric permutation-based t test. From these regions of significant difference, mean MTR in patients and healthy controls were extracted and used to calculate a relative percentage difference, which was defined as the threshold for demyelination. (B) Classification of cortical demyelinated voxels in patients. We then calculated the relative difference between the MTR value of any given cortical voxel in patients and the average MTR value of all voxels in healthy controls localized at the same distance than the given voxel from the external CSF. If this difference was greater than the previously calculated threshold, that cortical voxel was classified as "demyelinated", otherwise as "normally myelinated". As a result, we generated a map of cortical demyelination for each patient at each time-point

(iii) From these maps, we calculated the index of cortical remyelination, defined as the percentage of cortical voxels classified as demyelinated at baseline which were classified as normally myelinated at the second time-point, and the index of cortical demyelination, defined as the percentage of cortical voxels classified as normally myelinated at baseline which were classified as demyelinated at the second time-point. Finally, we calculated the index of cortical demyelination at baseline, defined as the percentage of cortical voxels classified as demyelinated at the first time point compared to healthy controls. To minimize partial volume effect, both indices were calculated for the cortical section included between the 25% and the 75% of the relative distance between CSF and WM.

[11C]PiB PET-derived indices of myelin content changes in white matter lesions

DVR maps of patients were used to compute individual maps of WM lesional myelin content changes as previously described[10]. The main steps of the analysis are briefly summarized below: (i) We first calculated a relative percentage threshold based on the relative difference between the mean DVR of WM lesions in patients with MS and the mean DVR of the WM of healthy controls. Then we compared the DVR of each given voxel in WM lesions with the mean DVR of all the voxels of HC localized at the same distance from the CSF (therefore potentially affected to the same extent by partial volume). If the difference between these two values was greater

than the previously calculated relative percentage threshold, that given voxel in patients was classified as demyelinated.

(ii) From the obtained maps, we generated (a) an individual map of demyelinating voxels at follow-up, defined as WM lesional voxels classified as normally myelinated at baseline and as demyelinated at follow-up; and (b) an individual map of remyelinating voxels at follow-up, defined as lesional voxels classified as demyelinated at baseline and as normally myelinated at follow-up[10]. Due to the alteration of the blood-brain barrier which might affect PET quantification, gadolinium enhancing lesions were excluded from the analysis.

ii) From these maps, we calculated the index of WM lesion remyelination, defined as the percentage of lesional voxels classified as demyelinated at baseline which were classified as normally myelinated at the second time-point, and the index of WM lesion demyelination, defined as the percentage of lesional voxels classified as normally myelinated at baseline which were classified as demyelinated at the second time-point. Finally, we calculated the index of WM lesion demyelination at baseline, defined as the percentage of lesional voxels classified as demyelinated at the first time point compared to healthy controls.

Statistical analysis

Statistical analysis was performed using R (https://www.r-project.org/). P-values <0.05 were considered significant. Shapiro-wilk tests and visual inspection of the histograms were performed to evaluate variable distribution.

The association between the cortical and WM lesion indices of remyelination and between each index of myelin content change and disease duration was investigated with Spearman's rank correlation. This same test was employed to assess the association between each of the indices of myelin content change and clinical scores. Only the indices significantly associated with clinical scores in the univariate analysis were then included in the regression analysis.

Hierarchical linear regression models were then applied to assess the role of the indices of myelin content change in predicting EDSS and MSSS. Sex and disease duration were included as confounding variables (level 1), and the significant indices of myelin content change resulting from the univariate analysis as explanatory variables (level 2 and level 3). Adjusted-R2 (adj-R2) have been reported for level 1 and adj-R2 changes compared to the lower levels have been used to quantify the respective contribution of the index of cortical (Level 2) and WM lesion remyelination (Level 3) in explaining the variance of the clinical scores. Likelihood ratio tests

between levels have been used to assess the significance of the adj-R2 changes and the contribution of each predictor has been reported as beta coefficient obtained from levels 3.

Results

Demographic, clinical and radiological characteristics of participants are summarized in Table 1.

The mean MTR value in cortical areas of significant group difference was 33.32 ±1.14 percent unit (pu) in patients, and 38.68±0.27 pu in healthy controls (percent difference=13.8%).

Table 1: Demographic, clinical and radiological characteristics of patients and healthy controls

Demographic, clinical and radiological characteristics	Patients	healthy controls	P value
Number	19	7	-
Age (years), mean±SD	32.3 ± 5.6	31.4 ± 6.5	0.74
Sex, female/male	13/6	7/0	0.23
Disease duration (years), mean±SD	7.4 ± 5.8	-	-
Follow up, mean months (sd; range)	2.46 (0.9; 1-4) 9 patients = 1 or 2 months. 10 patients = 3 or 4 months		
Treatment (number of patients with no treatment/ first line treatment/ second line treatment)	4/9/6	-	-
EDSS, median (range)	2 (0-6)	-	-
MSSS, mean±SD	3.7 ± 1.94	-	-
T2 lesion load, cc, mean±SD	33.78 ± 20.13	-	-

Both the indices of cortical and WM lesion remyelination were characterized by a wide inter-patient variability (index of cortical remyelination range: 17%-45% of cortical voxels classified as demyelinated at baseline, thus 1-5% of the total cortical volume; index of WM lesion remyelination range: 8%-22 of total WM lesion volume) (Figure 8).

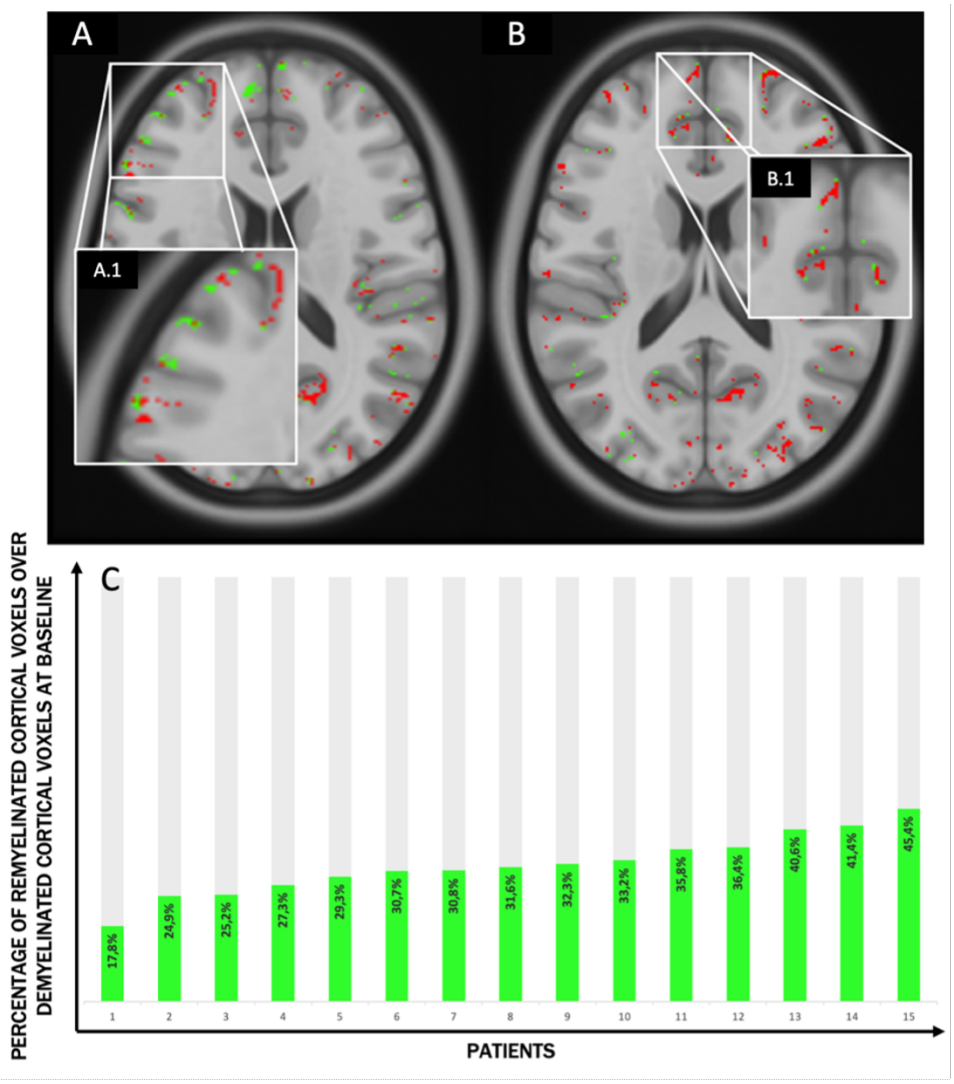


Figure 8. Examples of MTR-Derived maps of cortical myelin content changes in two patients with MS with similar extent of cortical demyelination at baseline (voxels in red) but with a very different extent of cortical remyelination (voxels in green), overload onto a T1-weighted scan in standard space. In A: 37-year-old woman, cortical demyelination at baseline = 10% of total cortical volume, cortical remyelination = 34,4% of cortical demyelinated voxels at baseline. In B: 27-year-old man, cortical demyelination at baseline = 10,7% of total cortical volume, cortical remyelination = 17,8% of cortical demyelinated voxels at baseline (magnifications of selected details of figures A and B are displayed in the inserts). In C, bar chart diagram showing the percentage of cortical remyelinated voxels over cortical demyelinated voxels at baseline in each patient.

A similar across-patients heterogeneity was found for both the index of cortical demyelination, (ranging between 2% and 6.5% of total cortical volume) and for the index of WM lesion demyelination (ranging between 8% and 20% of WM lesion volume). The index of cortical demyelination at baseline ranged between 2% and 12% and the index of WM lesion demyelination at baseline ranged between 34% and 65%.

No association was found between the cortical and WM lesion indices of remyelination (rho= 0.27, p=0.39) and between the cortical and WM lesion indices of demyelination (rho= 0.04, p=0.9). The index of WM lesion remyelination was associated with the index of demyelination

(rho=-0.57, p=0.027) but no association was found between the indices of cortical remyelination and demyelination (rho=-0.33, p=0.21).

A longer disease duration was associated with a greater amount of cortical demyelination at baseline (rho=0.71, p=0.002) and with a reduced amount of cortical remyelination (rho= -0.63, p=0.011), while no correlation was found between disease duration and the remaining indices of myelin content change (WM lesion remyelination, cortical and WM lesion demyelination, and WM lesion demyelination at baseline; p>0.64).

Higher indices of cortical and WM lesion remyelination were associated with a lower EDSS (cortical remyelination: rho= -0.49, p=0.05, WM lesion remyelination: rho= -0.61, p=0.014) and with a lower MSSS (cortical remyelination: rho= -0.62, p=0.011, WM lesion remyelination: rho= -0.61, p<0.01), while no correlation was found between clinical scores and the indices of cortical and WM lesion demyelination and demyelination at baseline (p>0.27). T2-w lesion load was not significantly associated with clinical scores (EDSS: rho=0.18, p=0.5; MSSS: rho=0.008, p=0.97). Regarding the hierarchical linear regressions, the addition of the index of cortical remyelination to the confounding factors (level 2) significantly improved both models (EDSS: adj-R2 change=+0.14, p=0.023; MSSS: adj-R2 change=+0.19, p=0.003). The subsequent inclusion of the index of WM lesion remyelination (level 3) further increased the quality of the predictions (EDSS: adj-R2 change=+0.15, p=0.032; MSSS: adj-R2 change= +0.35, p<0.001). Overall, the model which included sex, disease duration, the indices of cortical and WM lesion remyelination (Level 3) accounted for 68% (p=0.002) and 70% (p=0.0015) of the variance of EDSS and MSSS, respectively (Table 2).

Table 2: beta coefficients and p value of each predictor included in the regression models.

CLINICAL SCORE	PREDICTORS	LEVEL 1 Beta (p value)	LEVEL 2 Beta (p value)	LEVEL 3 Beta (p value)	LEVEL1 R2-adj (p value)	LEVEL2 R2-adj (p value)	LEVEL3 R2-adj (p value)
EDSS	DISEASE DURATION	0.70 (0.037)	0.21 (0.53)	0.31 (0.26)	0.39 (p=0.02)	0.53 (p=0.004)	0.68 (p=0.002)
	SEX (M)	0.52 (0.10)	0.42 (0.13)	0.24 (0.32)			
	CORTICAL REMYELINATION	-	-0.76 (0.04)	-0.60 (0.05)	-		
	WM LESION REMYELINATION	-	-	-0.57 (0.03)	-	-	
MSSS	DISEASE DURATION	-0.03 (0.91)	-0.42 (0.18)	-0.32 (0.13)	0.16 (p=0.2)	0.35 (p=0.004)	0.70 (p=0.001)
	SEX (M)	0.49 (0.08)	0.4 (0.10)	0.19 (0.24)			
	CORTICAL REMYELINATION	-	-0.62 (0.06)	-0,45 (0.04)	-		
	WM LESION REMYELINATION	-	-	-0,64 (0.002)	-	-	
CLINICAL SCORE	PREDICTORS	LEVEL 1 Beta (p value)	LEVEL 2 Beta (p value)	LEVEL 3 Beta (p value)	LEVEL1 R2-adj	LEVEL2 R2-adj	LEVEL3 R2-adj
EDSS	DISEASE DURATION	0.70 (0.037)	0.64 (0.06)	0.67 (0.07)	0.39 (p=0.02)	0.37 (p=0.04)	0.32 (p=0.09)
	SEX (M)	0.52 (0.10)	0.50 (0.12)	0.46 (0.18)			
	CORTICAL DEMYELINATION	-	0.25 (0.42)	0.24 (0.46)	-		
	WM LESION DEMYELINATION	-	-	0.13 (0.68)	-	-	
MSSS	DISEASE DURATION	-0.03 (0.91)	-0.04(0.88)	-0,19 (0.94)	0.10 (p=0.2)	0.03 (p=0.37)	0.007 (p=0.43)
	SEX (M)	0.49 (0.08)	0.47 (0.10)	0.42 (0.16)			
	CORTICAL DEMYELINATION	-	0.08 (0.78)	0,04 (0.88)	-		
	WM LESION DEMYELINATION	-	-	-0,23 (0.41)	-	-	

Chapter 3.2 - Cortical remyelination prevents cortical atrophy and disability progression: a multicenter, retrospective, longitudinal study

Introduction:

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) with inflammatory and degenerative components affecting both the white (WM) and the grey matter (GM). The key neuropathological hallmarks of MS include demyelination, inflammation, astrocytic gliosis, and neurodegeneration, which is considered the main pathological substrate of clinical progression (Mahad, et al., 2015). Among these, myelin loss is considered the cornerstone of the pathophysiology of MS and the dynamics of myelin content change are thought to have a relevant impact on neurodegeneration and disability all along the course of the disease (Correale , et al., 2019) (Lubetzki, et al., 2020) (Kiljan, et al., 2020).

Endogenous myelin repair in the central nervous system is a physiological response to a demyelinating event mediated by a population of adult brain resident progenitor cells (oligodendrocyte progenitor cells or OPCs), which migrate into areas of demyelination and differentiate into actively myelinating oligodendrocytes (Franklin & Goldman, 2015) (Zawadzka, et al., 2010).

While in experimental models remyelination is usually extensive, neuropathological studies have revealed that the process of myelin repair in white matter lesions has been shown to be sparse or ineffective in the majority of people with MS (Patrikios, et al., 2006). Furthermore, it has been shown that the extent of white matter lesion remyelination, which is highly variable across patients but can be extensive particularly in the early stages of this condition, tends to decrease as disease progresses, eventually failing and resulting in chronically demyelinated lesions (Patrikios, et al., 2006) (Patani, et al., 2007) (Franklin & Goldman, 2015). In addition, post-mortem studies have indicated that the remyelination process is also characterized by a significant variability between different lesions of the same patient, suggesting the presence of local factors influencing the extent of myelin repair (Goldschmidt, et al., 2009). So far, only few neuropathological studies have investigated the process of remyelination occurring in the cortex of people with MS. These studies, in addition to confirming that even in cortical regions the extent of remyelination varies considerably across patients, have provided the notable evidence that myelin repair is significantly more extensive in cortical than in white matter lesions (Chang, et al., 2012) (Werkman, et al., 2021) (Albert, et al., 2007).

Although this early neuropathological evidence points to the cortex as a particularly relevant region for remyelination in MS, a comprehensive in-vivo description of cortical remyelination and its determinants in people with MS, as well as a full understanding of the impact of this process on clinical progression are not yet available.

Among the imaging techniques proposed to quantify myelin content changes at the cortical level in patients with MS, magnetization transfer ratio (MTR), an imaging metric sensitive to myelin content changes in MS (Schmierer, et al., 2004) has been shown to be lower in cortical lesions than in normally myelinated cortex (Chen, et al., 2013) and has been successfully employed to generate maps of myelin loss and repair in cortical grey matter (Derakhshan, et al., 2014)(Lazzarotto, et al., 2022). The application of this technique to explore myelin loss and repair in cortical regions is supported by the recently introduced notion of a relatively higher MTR specificity for myelin in cortical grey matter areas than in white matter regions (Moccia, et al., 2020), where the MTR signal is significantly affected by processes other than myelin content changes, including astrogliosis, oedema and inflammation.

In this longitudinal retrospective multicenter study, we generated patient-specific MTR derived maps of cortical myelin content changes to provide the first comprehensive description of myelin loss and repair at the cortical level in a large cohort of people with all forms of MS. Our objective was to identify the key factors impacting the extent of these processes in MS and to explore the relevance of cortical myelin loss and repair in predicting cortical atrophy after one year and clinical progression after five years.

Methods:

Subjects and study design:

One-hundred-eighty patients who met the 2010 diagnostic criteria of MS (Polman, et al., 2011) were followed-up in four European centres of the MAGNIMS consortium (Graz, Milan, Paris, Siena) and enrolled in this retrospective longitudinal clinico-radiological protocol. Patients were selected according to the following criteria: (a) age range, 18–65 years; (b) no history/evidence of neurologic or psychiatric disorders other than MS; (d) MRI including MTI at baseline and at one year follow-up. Ninety-two sex- and age-matched healthy controls (HC), selected according to the following criteria, were also included in the study: (a) no history of neurological or psychiatric disorders; (b): good quality MRI at baseline; (c, optional): a second MRI including MTI (retest MRI) acquired after the baseline scan independently of the inter-scan interval.

All patients with MS were clinically assessed using the Expanded disability status scale (EDSS) (Kurtzke, 1983) at baseline, after one year (mean 12.5 ± 5.5 months), and again after 5 years

(mean 61.3 ± 7.1 months) of study entry. An increase of at least 0.5 points in the EDSS at one and five years compared to baseline EDSS was defined as EDSS worsening at 1 year and at five years respectively. All patients underwent the same MRI protocol at baseline and after one year of study entry. All healthy controls were clinically assessed at baseline and underwent the same imaging protocol as patients at study entry, while a subgroup repeated an identical imaging protocol a second time (10 from Paris and 7 from Siena).

Due to the suboptimal quality of some of the collected MRI scans, the images of 40 patients and 8 healthy controls were not retained for the study, leaving a total of 140 patients and 84 healthy controls with at least a good-quality MRI scan at baseline for further analysis (Graz: 54 patients and 38 healthy controls, Paris 60 patients and 39 healthy controls, Siena 26 patients and 7 healthy controls) (Table 1).

All subjects gave informed, written consent before the study, which was approved by the local Ethics Committees of each participating centre.

Image acquisition:

In all cohorts, images were acquired on 3T scanners and included the following sequences: 3D-T1-weighted sequence for anatomical imaging and volume analysis, T2-weighted sequences (Paris, Siena) and fluid attenuation inversion recovery (Graz) for white matter (WM) lesion segmentation, and 3D gradient-echo with (Mton) and without (Mtoff) magnetization transfer for cortical myelin content analysis

MRI post-processing

MR anatomical image processing

Native space

In patients, WM lesions were manually contoured on the T2-w /FLAIR images at baseline and at 1 year by the same neurologist (A.L.) using Jim (v6.0, http://www.xinapse.com/), revised and manually corrected if needed by an expert neurologist (B.B.), and transformed into binary masks. T2-w/FLAIR images were aligned to the corresponding 3D-T1-weighted scans using a rigid registration obtained with FLIRT, part of FSL (http://fsl.fmrib.ox.ac.uk/), and the derived transforms were then employed to register lesion masks onto 3D-T1-weighted scans. After "lesion-filling" in patients (Chard DT, 2010), cortical GM, WM, ventricles and peri-pial cerebrospinal fluid (pCSF) were segmented in all subjects at both time-points on 3D-T1-weighted scans using Freesurfer (https://surfer.nmr.mgh.harvard.edu), then manually corrected (A.L.) and revised by an expert neurologist (B.B.). For each subject, a relative distance map

from the pCSF and from the WM was generated using FSL (http://fsl.fmrib.ox.ac.uk/fsl/), applying the following formula [distance from pCSF/(distance from pCSF + distance from WM)].

Standard space

For each patient, the T1- weighted images acquired at the two time-points were aligned to each other to create a "halfway space" following the longitudinal images processing of Freesurfer (Reuter et al., 2012). The derived images, and the baseline T1-w images in healthy controls, were normalized to the standard space (MNI) using a non-linear registration with ANTs (http://stnava.github.io/ANTs). Derived transformations were then applied to move all images (including T2-w/FLAIR lesion masks, cortical GM masks and distance maps) from native space to standard space (MNI152), passing through the halfway space in patients (Figure 1).

Calculation of cortical grey matter volume change over one year in patients

Cortical GM volume at baseline and at 1 year were obtained using Freesurfer and normalized for the total intracranial volume (Table 1). Cortical GM masks in the "halfway space" of each time-point were used as ROI inputs for the Jacobian integration method to calculate the cortical grey matter volumetric change between baseline and one year (Nakamura, et al., 2013). To this purpose, we applied a non-linear registration with ANTs between linearly aligned time-points using the 3D-T1-weighted images to generate a deformation field describing the local displacement at each voxel that best aligned the two images. We computed the Jacobian of the deformation field at each single voxel, and we then extracted the determinant of the Jacobian, reflecting the magnitude of local volume change at each voxel as a percentage (Elliott et al, 2018). Averaging all the cortical voxels of each patient, we obtained a single value representing the mean magnitude of volume change for the whole cortex (Figure 1).

MT image processing

Generation of MTR maps

In all subjects, MTR maps were generated in native space using the equation MTR=(Mtoff-Mton)/Mtoff and rigidly aligned to the corresponding T1-w scans using FLIRT (http://fsl.fmrib.ox.ac.uk/). Using the previously derived transformations, MTR on T1 space were moved to MNI152, passing through the halfway space in patients (Figure 9 A).

Calculation of centre-specific thresholds for cortical GM demyelination at each time-point

Regions of significant difference in cortical MTR between patients and healthy controls of each centre were identified using a voxel-wise permutation-based two-sample t-test adjusted for age

and sex, thresholded at $p<0.05$, after threshold-free cluster enhancement correction. In these significant regions, we computed the MTR mean relative difference between patients and healthy controls [(mean MTR in healthy controls – mean MTR in patients)/mean MTR in healthy controls], which was then employed as centre-specific threshold to classify demyelinated voxels in patients (Figure 9 B). At the end of this step, we obtained three separate thresholds, one for each center, expressed in the form of percentage of difference in MTR between patients and healthy controls in cortical regions of significant difference between the two groups.

Generation of individual cortical maps of demyelination at each time-point and calculation of patient-specific indices of cortical myelin content change:

In each patient at each time-point, we then calculated the relative difference between the MTR value of any given cortical voxel and the average MTR value of all voxels in healthy controls of the same center localized at the same distance than the given voxel from the pCSF (thus suffering from the same degree of partial volume effect). If this difference was greater than the previously calculated center-specific threshold, that cortical voxel was classified as "demyelinated", otherwise as "normally myelinated". As a result, we generated a map of cortical demyelination for each patient at each time-point (Figure 9 C).

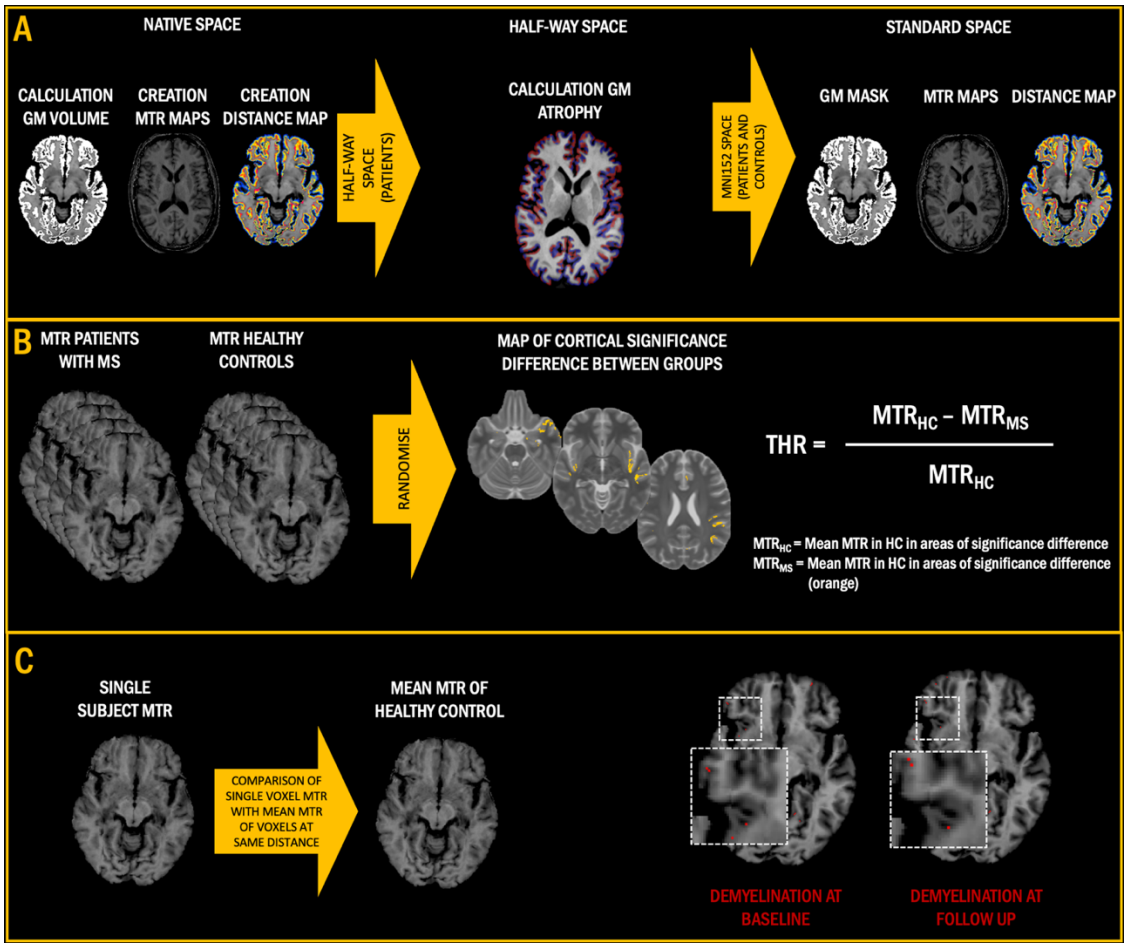


Figure.9: Simplified method flowchart. (A) cross-sectional GM volume, MTR maps and distance maps were created in native space and passing throw the half-way space in patients were normalized to standard space. In half-way space, t1w images were used to calculate cortical GM atrophy using the Jacobian integration method. (B) Definition of a threshold to identify cortical demyelinated voxels in patients compared to healthy controls. Regions of significant group differences between MTR maps of patients with MS and healthy controls were identified through voxel-wise nonparametric permutation-based t test. For each center, from these regions of significant difference, mean MTR in patients and healthy controls were extracted and used to calculate a relative percentage difference, which was defined as the threshold for demyelination. (C) Classification of cortical demyelinated voxels in patients. We then calculated the relative difference between the MTR value of any given cortical voxel in patients and the average MTR value of all voxels in healthy controls localized at the same distance than the given voxel from the external CSF. If this difference was greater than the previously calculated threshold, that cortical voxel was classified as "demyelinated", otherwise as "normally myelinated". As a result, we generated a map of cortical demyelination for each patient at each time-points

Comparing the patient-specific maps of cortical demyelination at the two time-points, we then generated for each patient a map of cortical remyelination and a map of dynamic demyelination. Each cortical voxel classified as demyelinated at baseline which was then classified as normally myelinated at follow-up was defined as "*remyelinated*", while each cortical voxel classified as normally myelinated at baseline which was then identified as demyelinated at follow-up was considered as "*dynamically demyelinated*".

Only the cortical section of these maps included between the 25% and the 75% of the relative distance between the CSF and WM was retained for further analysis to reduce partial volume

effect. From the final maps, we extracted three indices of cortical myelin content change: (i) the index of cortical demyelination at baseline, defined as the percentage of cortical voxels classified as demyelinated at baseline over total cortical volume; (ii) the index of cortical dynamic demyelination, defined as the percentage of cortical voxels classified as normally myelinated at baseline which were then identified as demyelinated at the second time-point; and (iii) the index of cortical remyelination, defined as the percentage of cortical voxels classified as demyelinated at baseline which was then identified as normally myelinated at the second time-point (Figure 10).

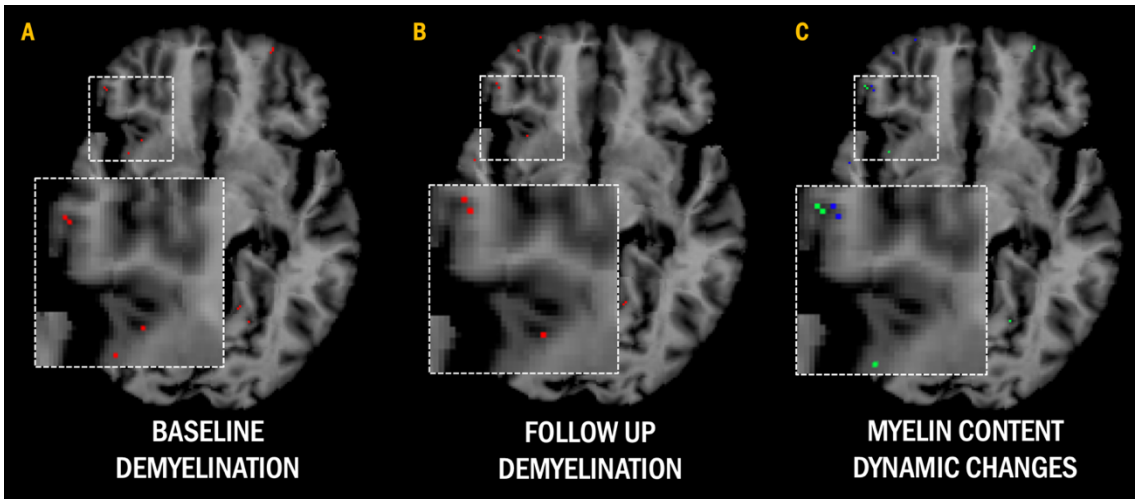


Figure.10: MTR based myelin content maps. In A and B are highlighted in red the voxels which are classified as demyelinated compared to healthy controls at baseline and at follow respectively. In C are highlighted in blue the cortical voxels which were normal at baseline, and which were classified as demyelinated at follow up (dynamic demyelination); in green the demyelinated voxels at baseline which recovered a normal MTR signal at follow up (remyelination).

Probability of myelin content changes and distance from peri-pial CSF:

For each patient, the three individual maps of cortical myelin content change were overlaid onto the distance map to calculate the probability of cortical regions to be demyelinated at baseline, dynamically demyelinating or remyelinating over the follow-up, given their distance from pCSF and WM. More in details, the probability of the cortex to be demyelinated at baseline was calculated for each given distance of the distance map as the ratio between the number of voxels classified as demyelinated at baseline at that given distance from the CSF and the total number of cortical voxels localized at that specific distance from the CSF. The probability of cortical voxels to be dynamically demyelinating over the follow-up at a given distance from CSF were calculated as the number of voxels classified as demyelinating over the follow-up at that distance from peri-pial CSF normalized with the number of cortical voxels classified as normally myelinated at baseline at the same distance. Finally, the probability of cortical voxels to remyelinate over the follow-up at a given distance from peri-pial CSF was obtained with the

number of voxels classified as remyelinating over the follow-up at a given distance from peri-pial CSF normalized with the number of lesional voxels classified as demyelinated at baseline at the same distance.

Evaluation of misclassification errors

To cross-validate the classification of each voxel, we performed a leave-one-out test on healthy controls for whom an MT acquisition was available as a retest.

Each healthy control at each time-point (test and re-test) was singularly treated as a patient and compared to the other subjects of the same centre to create two maps of "cortical demyelination", the first generated from the test scan and the second from the re-test scan, following the same procedure applied in patients with MS. From the comparison of these maps, we generated a map of "cortical dynamic demyelination" and of "cortical remyelination" and then we extracted three fictitious indices of cortical myelin content change (index of cortical demyelination at baseline, index of dynamic cortical demyelination, index of cortical remyelination) which reflected the misclassification error in the corresponding indices in patients with MS.

Statistical analysis

Statistical analysis was performed using R (https://www.r-project.org/). Two-sided P value <0.05 was considered significant.

Shaphiro-Wilk test and visual analysis of histograms were used to test the normality of the data. Associations between clinical and imaging variables (age, disease duration, the three indices of myelin content and grey matter cortical atrophy) and differences in imaging variables between groups (sex, treatment, EDSS worsening vs not worsening at one and five years, and MS clinical phenotypes) were estimated with linear regression models adjusted for center and time between scans.

Linear and logistic regression models were respectively applied to predict cortical GM atrophy at one year and EDSS worsening at five years. In these models, center, time between scans, sex, disease duration, MS clinical phenotypes and WM lesion volume were included as covariates and the three indices of myelin content change were individually and together included as explanatory variables. To test the hypothesis of a different effect of cortical remyelination according to the amount of cortical demyelination at baseline and the disease duration, in additional models we included the interaction terms with these two variables.

Beta coefficients and odds ratios (OR) are reported along with their p values for each predictor, as appropriate. Adjusted-R^2 and areas under the curve (AUC) are reported as an estimation of the quality of the prediction for linear and logistic models respectively. Likelihood ratio test (LRT) were applied to test the difference between models with and without interaction terms.

Results

Out of the 140 patients with MS included in this study, 37 presented a clinically isolated syndrome suggestive of MS (CIS), 71 a relapsing-remitting MS (RMS) and 32 a progressive MS (PMS; 5 secondary progressive MS, 27 primary progressive MS). Twenty patients experienced an EDSS worsening and 120 were clinically stable or improved at one year. Sixty-seven patients experienced a EDSS worsening at five years, 69 were clinically stable or improved and for 4 patients clinical data at five years were not available (see Table 3 for detailed demographic characteristics).

The mean MTR value in cortical areas of significant group difference was 33.26 ±1.14 percent unit (pu) in patients, and 38.76±0.27 pu in healthy controls, with a resulting mean percent difference of 14.2% (Paris: 14.6%, Graz: 14.1%, Siena: 13.9%).

A misclassification error < 1% was obtained from the leave-one-out test performed on healthy controls in both cohorts of Paris and Siena. Specifically, out of all the cortical voxels, only 0.47% and 0.52% were classified as demyelinated at baseline, 0.21% and 0.23% as dynamically demyelinated and 0.3% and 0.38% as remyelinated in the Paris and Siena cohorts respectively.

TABLE 3.	MS PATIENTS	RMS	PMS	HEALTHY CONTROLS
N°	140	108	32	84
N° from each CENTER	GRAZ: 54 PARIS: 60 SIENA: 26	GRAZ: 54 PARIS:28 SIENA:26	GRAZ: 0 PARIS: 32 SIENA: 0	GRAZ: 38 PARIS: 39 SIENA: 7
WOMEN/MEN	96/44	80/28	16/16	55/29
AGE (mean ± sd)	38.2 ± 12.2	35.13 ± 10.44	51.44 ± 9.75	34.3 ± 10.97
DISEASE DURATION AT BASELINE (months) (mean ± sd)	64.81 ± 67.21	53.43 ± 62.5	102.82 ± 71.01	-
MRI DELAY (months) (mean ± sd)	12.52 ± 5.5	11.7 ± 6.66	12.72 ± 3.4	-
EDSS AT BASELINE (median and range)	2.0 [0.0 - 7.5]	2.0 [0.0 - 6.0]	6.0 [3.0 - 7.5]	-
EDSS AT 1 year (median and range)	2.0 [0.0 - 7.0]	2.0 [0.0 - 6.0]	5.0 [3.0 - 7.0]	-
EDSS AT 5 years (median and range)	2.0 [0.0 - 8.0]	2.0 [0.0 - 7.5]	6.0 [3.0 - 8.0]	-
TYPE OF TREATMENT (N°; NT= not treated; FL=first line treatment, SL=second line treatment)	NT= 85; FL= 41; SL= 14	NT= 31; FL= 31; SL= 9	NT= 25; FL= 2; SL= 5	-
T2 LESION VOLUME (mm3; mean ± sd)	14964 ± 18326.39	14898 ± 19840.64	15183± 12206.81	-
CORTICAL ATROPHY (cc, mean ± sd)	0.002819 ± 0.0097	0.0043 ± 0.0092	-0.0023 ± 0.0093	-

Cortical myelin loss and repair are heterogenous across patients with MS

The indices of cortical myelin content changes were characterized by a wide inter-subject variability, with the percentage of demyelinated voxels at baseline ranging from 0.4% to 20% of total cortical voxels, the percentage of dynamically demyelinated voxels ranging from 0.3% to 20% of normally myelinated voxels at baseline and remyelinated voxels ranging from 14% to 95% of demyelinated cortical voxels at baseline (Figure 11 A).

Considering separately each of the MS clinical phenotypes, we confirmed the same heterogeneity: in RMS from 0.7% to 20% (demyelination at baseline), from 0.3% to 20% (dynamic demyelination) and from 14% to 95% (remyelination); in PMS from 0.4% to 14% (demyelination at baseline), from 0.4% to 16% (dynamic demyelination) and from 26% to 89% (remyelination).

In subpial regions, demyelination is more severe and remyelination fails

Sub-pial cortical regions showed a greater amount of demyelination at baseline, a greater amount of dynamic demyelination, and a lower amount of remyelination compared to the rest of the cortex. We also found that, with increasing distance from peri-pial CSF, the mean probability of cortical remyelination increased from 10% to 49%, the mean probability of cortical demyelination at baseline decreased from 44% to 10%, and the mean probability of cortical dynamic demyelination over the follow up decreased from 39% to 1% (Figure 11 B-D).

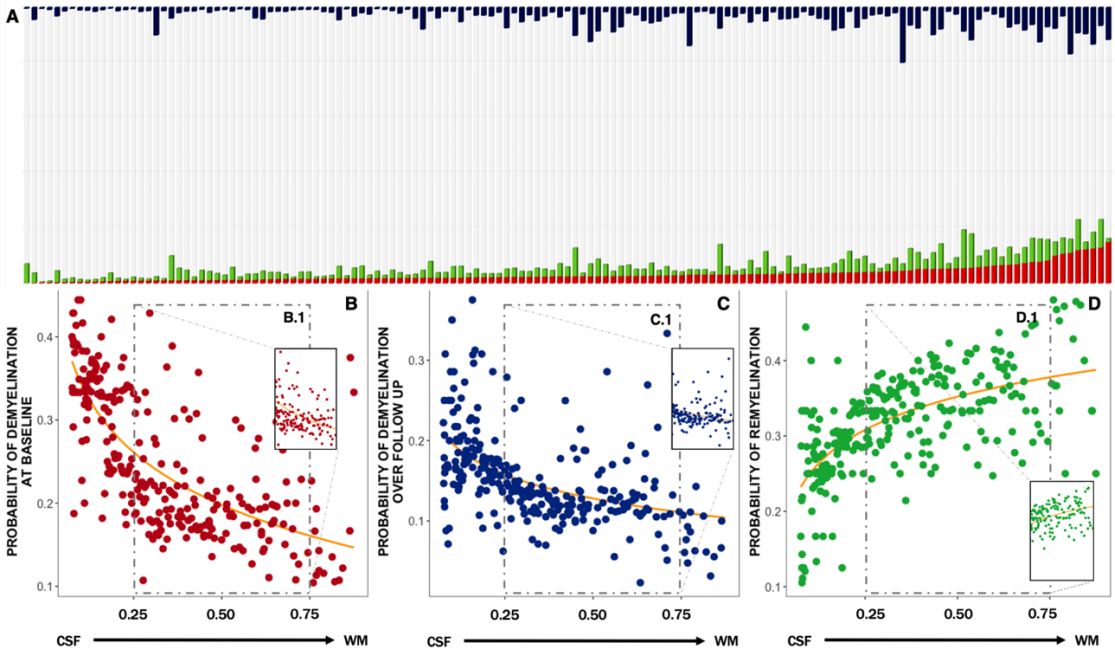


Figure.11: the three indices of cortical myelin content change are largely heterogeneous and are affected by the distance from CFS. In A is represented a bar plot in which each bar represents a patient's cortex: in red are highlighted the voxels demyelinated at baseline and at follow up, in green the cortical voxels which were demyelinated at baseline and remyelinated at follow up, and in blue the cortical voxels which were normal at baseline and demyelinated at follow up. In B-D are shown the mean probabilities of cortical myelin content change: demyelination at baseline (B), dynamic demyelination (C) and remyelination (D) according to distance from peripial CSF. A.1, B.1, C.1 represent the spatial distribution of the mean probability for the portion of the cortex from which the indices of myelin content change were extracted, and in which the cortical gradients were calculated to reduce the effect of partial volume.

Longer disease duration is associated with a greater amount of cortical demyelination in patients with all forms of MS

A greater amount of cortical demyelination at baseline was associated with a greater amount of dynamic demyelination (β =0.53, p=6.09e-10), whereas no significant association was found between the indices of cortical demyelination (at baseline and dynamic) and the index of remyelination (p=0.32 and p=0.93 respectively).

A longer disease duration was associated with a greater amount of demyelination at baseline (β =0.52, p= 2.08e-07) and with a greater amount of dynamic demyelination (β = 0.34, p= 0.002). No significant correlation was found between disease duration and the index of cortical remyelination (β = 0.08, p=0.33) except for the RMS population, in which longer disease durations were associated with cortical remyelination failure (β =-0.23, p=0.02; PMS p=0.15). No correlation was found between the indices of cortical myelin content change and age (p>0.34).

A greater amount of cortical demyelination at baseline was present in male patients (Beta=0.14, p=0.03) compared to women, in both RMS and PMS compared to CIS (Beta=0.25, p=0.006 and Beta=0.34, p=0.006, respectively). Similarly, a greater amount of dynamic demyelination was present in RMS and PMS patients compared to CIS (Beta=0.26, p=0.003 and Beta=0.27, p=0.02, respectively). No significant difference between women and men nor MS clinical phenotypes was found.

A lower amount of cortical remyelination was found in patients treated with first-line treatments compared to non-treated patients (Beta=-0.21, p=0.013). No other significant group difference was found for all the other indices.

Increased cortical remyelination reduces the probability of cortical GM atrophy developing over one year

A failure in cortical remyelination significantly predicted a greater GM atrophy at one year (β =-0.20, p=0.019). The whole model, including age (β =0.11, p=0.091), sex (Male, β =-0.07, p=0.37), disease duration (β =0.02, p=0.79), T2 lesion volume (β =-0.07, p=0.5) and the MS clinical phenotype (β =- 0.15, p=0.14) explained 13% of the variance of GM volume change developing over one year (adjusted-R^2=0.127, p= 0.0012).
In the regression model including the three indices of cortical myelin content change together, both a failure in remyelination (β =0.18, p=0.03) and an increase in the amount of dynamic demyelination (β =0.23, p=0.024) predicted a greater GM atrophy at one year, independently of the amount of demyelination at baseline (p=0.14), age (p=0.10), sex (p=0.25), disease

duration (p=0.67), WM lesion volume (p=0.52) and MS clinical phenotype (p=0.13), explaining 14% of the GM atrophy variance (adjusted-R^2=0.142, p=0.0015) (Figure 12).

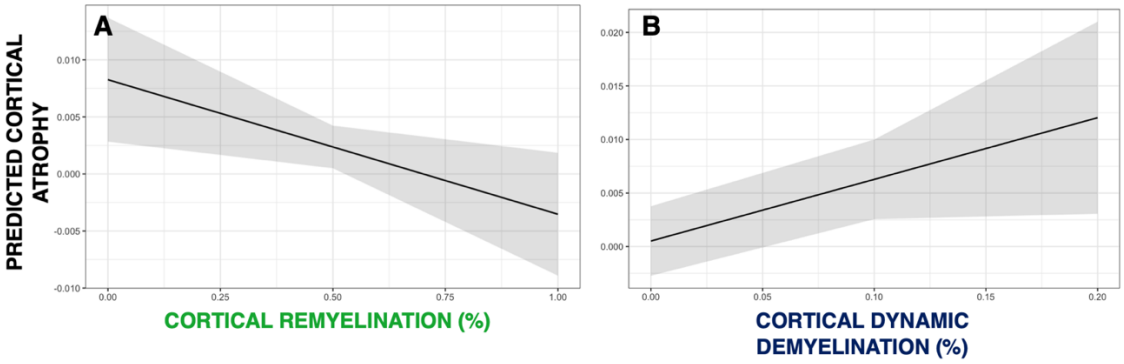


Figure.12: prediction of cortical GM atrophy. In A it is s displayed how the increase in the amount of cortical remyelination predicted lower cortical GM atrophy. In B it's displayed how the increase in the amount of cortical dynamic demyelination predicted higher cortical GM atrophy.

Cortical remyelination prevents EDSS worsening at 5 years in patients with shorter disease duration and limited amount of demyelination at baseline

A greater amount of cortical demyelination at baseline was significantly associated with a higher probability of EDSS worsening at five years independently of disease duration, sex, T2 lesion volume at baseline and GM atrophy developing over 1 year (p>0.12) (Table 2).Similarly, a greater amount of dynamic demyelination was associated with a higher probability of EDSS worsening at five years (OR=1.14, p=0.05) independently of disease duration, sex, T2 lesion volume at baseline and GM atrophy developing over 1 year (p>0.1).

While no association was found between the index of cortical remyelination and EDSS worsening at 5 years in the whole patient cohort, the same regression model including the interaction terms showed a significant role of the interaction between cortical remyelination, disease duration and cortical demyelination at baseline in the prediction of long-term disability progression (p=0.026, AUC=0.75, LRT p= 0.0007). In particular, a greater amount of cortical remyelination was significantly associated with a lower probability of EDSS worsening at five years only in the subgroup of patients with an amount of cortical demyelination at baseline lower than 8.5% of total cortical volume (corresponding to the mean value of cortical demyelination at baseline in our cohort) and with a disease duration shorter than 5 years (Figure 13 A). In this subgroup of

patients, the index of cortical remyelination significantly contributed to the prediction of EDSS worsening at five years (OR=0.90, p=0.004, AUC=0.81) independently of sex, GM atrophy developing over one year, T2 lesion volume and MS clinical phenotype (p>0.14). In these patients, an increase of 30% of cortical remyelination reduced by 43.5% the risk of EDSS worsening at five years (Figure 13 B).

Conversely, in patients with MS with more than 8.5% of cortical voxels classified as demyelinated at baseline and a disease duration longer than 5 years, no significant predictive value of cortical remyelination on EDSS worsening was found.

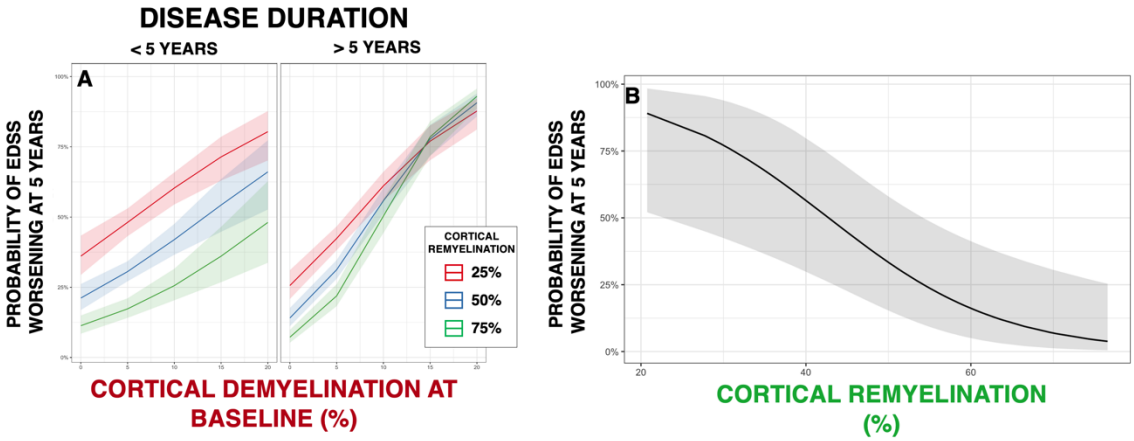


Figure.13: predicted probabilities of EDSS worsening at five years accordingly to different amount of demyelination at baseline and disease durations. In A, predicted probabilities of EDSS worsening at five years at three different amounts of cortical remyelination (mean +/- standard deviation), accordingly to different amounts of demyelination at baseline and in MS patients with disease duration longer and shorter than five years. In B the predicted probabilities of EDSS worsening accordingly to cortical remyelination, in MS population with disease duration shorter than five years and with limited amount of demyelination at baseline (<8.5%).

Chapter 4 – Discussion

Although more than 15 therapies are available to treat MS, the disease is far from being controlled or "cured". In particular, available immunotherapies have proven to be unable to delay or stop the accumulation of clinical disability over time in patients with MS, and the prevention of disease progression remains a significant unmet need (Reich et al., 2018). (Faissner, et al., 2019). Therefore, new therapies that protect neuronal structure and function, or that promote CNS repair are being actively sought to improve the management of MS (Faissner, et al., 2019) (Faissner & Gold, 2019) (De Angelis, et al., 2018).

Remyelination is an area in which new drugs have targeted mediators of myelination (Plemel, et al., 2017), whereas regeneration lies significantly behind and still awaits the promise of stem cells-based treatment. Although no promyelinating drug has been approved to date, the advances in understanding the underlying biology of remyelination and the large number of clinical trials of new therapeutics targeting the process of myelin regeneration offer promising therapeutical perspectives for the near future.

At present, a major limitation in the evaluation of these novel compounds is the absence of informative *in vivo* biomarkers and imaging end-points. The present PhD works includes the results of two different studies. In the first one, conducted on a cohort of 15 patients with MS from Paris (which from now on will be referred to as the "Paris cohort") and presented in chapter 3.1 I have proposed a novel methodological process to evaluate in vivo cortical myelin repair. In the second one, that has been conducted on a large cohort of 140 patients with MS from the MAGNIM%S consortium (which from now on will be referred to as the "MAGNIMS cohort") and that has been presented in chapter 3.2, I have explored the clinical relevance of cortical remyelination over a long follow-up.

Chapter 4.1 - explore novel MTR-based method to measure myelin content changes at the cortical level in MS

In the first part of this section, I will first justify the choice we made to employ MTR over PIB-PET, a technique largely employed by my team in Paris to explore myelin content changes in the white matter, to investigate demyelination and remyelination at the cortical level in patients with MS. Then, I will discuss the advantages and the limits of the novel methodology I proposed to generate patient-specific maps of cortical myelin loss and repair.

In the evaluation of myelin content in in the white matter, PIB- PET allows a significant increase of both the sensitivity and specificity of the information obtained (Bodini, et al., 2021).

This technique allowed with reasonable approximation to explore the clinical relevance of the process of myelin repair in white matter lesions, and to minimize the interpretative errors resulting from the reduced specificity of other imaging methods used for the same purpose (Bodini, et al., 2016) (Ricigliano, et al., 2022).

Indeed, it is noteworthy that the non-invasive quantification of the PET signal produced by any radiotracer requires some technical assumptions.

Among these, the main necessary condition is the selection of a reference region that is devoid of the target under study. In MS, changes are diffuse and can affect virtually any area of the brain, preventing the use of pre-specified anatomical reference regions. An elegant technique to overcome this problem is represented by the supervised clustering algorithm that can identify non-pathological areas of the brain by comparing the radiotracer kinetics in different brain areas with those derived from a group of healthy individuals. (Veronese, et al., 2015) (Bodini, et al., 2016) (Bodini, et al., 2021). In the case of [^{11}C] PIB-PET, the reference region is represented by cortical gray matter and consequently, the quantification of the myelin content and myelin content change in this region is not possible.

In this context, to explore myelin content changes at the cortical level, we chose to use MTR, since this technique has been shown to be sensitive to myelin content changes in MS (Schmierer, et al., 2004), even at the cortical level (Chen, et al., 2013) and has already been successfully employed to generate maps of myelin loss in cortical GM (Derakhshan, et al., 2014).

In general, a common finding of all these histopathological studies concerns the specificity of MTR for myelin. Indeed, it is known that the signal obtained from this MRI sequence is affected by other processes than to myelin content changes, among all oedema, inflammation,

astrogliosis and axonal loss (Schmierer, et al., 2004) (van Waesberghe, et al., 1999). it is also known, however, that these processes, are less common at the cortical level (Chang, et al., 2012) (Peterson, et al., 2001) and that consequently the specificity for myelin is greater in that region, as demonstrated by Moccia and colleagues (Moccia, et al., 2020).

Using MTR, we have developed a new method to generate for the first-time individual maps of cortical myelin loss and repair, based on a voxel-wise comparison between patients and healthy controls to classify cortical voxels in normally myelinated, demyelinated-ing remyelinated.
However, while we were developing this new method, we were well aware of the potential impact of partial volume effect on our results using MTR in a relatively small region as the cortex. We have therefore implemented several strategies to minimize the impact of partial volume effect on our data. First, we employed distance maps to compare cortical voxels in patients with cortical voxels in healthy controls localized at the same distance from the CSF, therefore suffering from the same amount of partial volume effect. Secondly, we did not consider the inclusion of the regions closer to the CSF and closer to the WM sufficiently reliable since those two regions are more affected by the partial volume effect. As a consequence, we included in the analysis the central portion of the cortical ribbon, thus reducing the risk of over-classification in terms of demyelination and remyelination.

It is worth indicating that the method we have chosen has been selected over others, based on the excellent misclassification error rate we obtained in the validation phase. In particular, in the MAGNIMS cohort, 10 healthy controls from Paris and 7 from Siena presented a test-retest acquisition which allowed us to perform a leave-one-out test and to compare different voxel classification methods in terms of misclassification error rate. For instance, to classify a single voxel as demyelinated, we tested the Z-score analysis applying the formula [MTR in patients < Mean MTR in healthy controls – 1.97 standard deviations]. From this analysis the misclassification error obtained from the leave-one out test was superior to 5% for the voxels demyelinated at baseline. In contrast, the method used in my studies misclassified less than 1 % of cortical voxels as demyelinated at baseline and remyelinated in two different groups of healthy controls (Paris and Siena).

Chapter 4.2 - Cortical myelin repair prevents disability worsening and neurodegeneration

Cortical myelin loss and repair were found to be heterogeneous in our patients, both in the Paris and in the MAGNIMS cohort. This finding confirms for the first time in vivo the variable extent of cortical myelin content changes demonstrated in post-mortem MS brains (Strijbis, et al., 2017).
As previously discussed in the introduction (Chapter 1.2.3), one of the factors which seems to influence most myelin loss and repair is lesion location (Albert, et al., 2007) (Chang, et al., 2012) (Strijbis, et al., 2017). The finding that in the white matter periventricular lesions exhibit less extensive remyelination in comparison to subcortical ones (Goldschmidt, et al., 2009) (Patrikios, et al., 2006) (Tonietto, et al., 2022), and that in the grey matter subpial regions are characterized by greater amount of demyelination and microstructural damage (Haider, et al., 2016) than the rest of the cortex , confirms in vivo the key role of lesion localization in modulating the efficiency of myelin rapair .
In particular, in the white matter of the Paris cohort, using both [^{11}C] PIB-PET and MTR in another study of ours (Tonietto, et al., 2022), we confirmed the presence of a gradient of demyelination and remyelination in which the periventricular regions show a greater amount of demyelination and a less effective remyelination process. In the MAGNIMS cohort we demonstrated that these regional differences with regard to the efficiency of remyelination exist also at the cortical level. In particular, confirming the results obtained by Samson and colleagues (Samson, et al., 2014), we found that subpial regions present a greater amount of demyelination at baseline and a greater amount of dynamic demyelination compared with the rest of the cortex, with a mean probability of cortical demyelination decreasing as the distance from CSF increases (at baseline: from 44% to 10%; over the follow up: 39% to 1%) (figure 11 B and C). On the contrary, when we explored the distribution of the cortical myelin repair, we found that failure of cortical remyelination is more common in subpial regions compared the regions closer to the WM, resulting in an increasing mean probability of remyelination as the distance from CSF increases (from 10% to 49%) (figure 11 D).
Multiple hypotheses can be proposed to explain these gradients in demyelination and remyelination, both in the WM and in the cortex. Among these, there is certainly the preferential localization of demyelination near the ventricles in MS (Brownell & Hughes, 1962) (Dawson, 1916). The origin of the majority of white matter lesions is most certainly perivenular, and the high venous density in periventricular areas could explain why lesions in this location are particularly frequent (Haider, et al., 2016). The failure of remyelination in this area (Kolb, et al.,

2021), could be the consequence of recurrent demyelination episodes and/or the result of a persisting process of demyelination leading to an exhaustion of oligodendroglial progenitor or mature oligodendrocyte pools (Jäkel, et al., 2019) (Yeung, et al., 2019). However, other brain areas have a high venous density, but they do not seem to be as severely affected by demyelination as periventricular regions (Haider, et al., 2016). This observation led to the suggestion that focal lesions in multiple sclerosis occur at sites with high venous density, but they tend to be more persistent in areas of low oxygenation (Haider, et al., 2016) (Papadopoulou, et al., 2014) (Martinez Sosa & Smith, 2017) such as the frontal and occipital horns of the lateral ventricles (Mayer & Kier, 1991). Oligodendrocyte precursor cells are especially vulnerable to hypoxia (Martinez Sosa & Smith, 2017) and the reduced availability of oxygen in these areas might contribute to the observed remyelination failure. Another interesting hypothesis that has raised much attention over recent years is that soluble inflammatory factors could diffuse from the CSF into the surrounding regions of the brain (i.e. periventricular white matter and subpial cortex) to directly cause microstructural damage in affecting both neurons and oligodendrocytes (Dawson, 1916) (Alcázar, et al., 2000) (Wentling, et al., 2019), thus potentially contributing to the inhibition of myelin repair. However, despite the presence of lesions which do not seem to arise from a central vein but possibly from the ependymal surface of the ventricle itself in the periventricular white matter (Gilmore, et al., 2009) and in the thalami (Magliozzi, et al., 2022), the majority of periventricular lesions are situated around subependymal veins (Adams, et al., 1987). This suggests that the presence of these putative factors in the CSF, may not play a major role in WM lesion initiation, but instead play a key role on lesion fate. On the contrary, when considering the putative mechanisms of the lesion formation at the cortical level, the proximity to the CSF seems to assume a preponderant role. Increasing evidence suggest that meningeal inflammation plays an important role in cerebral cortical GM pathology in MS (Magliozzi, et al., 2010) (Magliozzi, et al., 2007) (Pirko, et al., 2007). Ectopic B cell follicle-like structures are detectable in the cerebral meninges of a large proportion (40%) of cases with SPMS (Howell, et al., 2011) and those patients displayed a greater global meningeal inflammation that was associated with increased GM pathology. Microglial activation, caused by the diffusion of inflammatory mediators from the meninges, could contribute to cortical pathology through the release of cytotoxic molecules (Howell, et al., 2011) with subsequent increase of demyelination. Interestingly, *in vitro* studies have also showed a toxic effect of the CSF of people with MS on cultured oligodendrocytes (Alcázar, et al., 2000) (Wentling, et al., 2019) leading to failure of remyelination through the induction of a pro-proinflammatory environment (El Behi, et al., 2017) which is hostile for repair (Larochelle, et al., 2021) (Heß, et al., 2020).In this scenario, the demonstration of a cortical gradient of remyelination is of particular relevance, both at the cortical

level, where putative factors from the meninges via the CSF on the one hand increase the likelihood of demyelination and on the other seem to impair myelin repair mechanisms, but also at the WM level where, among the various mechanisms discussed, proximity to the CSF seems to play a decisive role at least in determining the lesion fate (Tonietto, et al., 2022).

In the Paris Cohort we found no significant correlation between the extent of cortical and WM lesion myelin repair and between the indices of cortical demyelination and remyelination. This evidence supports the notion that the process of myelin repair has different characteristics in the white matter and in the cortex, even in the same patient, and confirms in vivo of previously obtained neuropathological data (Albert, et al., 2007) (Chang, et al., 2012) (Strijbis, et al., 2017). This difference in the efficiency of myelin repair between white and grey matter could result from a more permissive environment for repair in cortical lesions compared to WM plaques. In particular, cortical regions might be less affected by inhibitory cues from astrocytes, microglia or extra-cellular matrix factors (Werkman, et al., 2021) and seem to be characterized by an increased availability of oligodendrocyte progenitor cells compared with white matter areas (Albert, et al., 2007) (Strijbis, et al., 2017).

The evidence of a pattern of chronically demyelinated MS lesions either being devoid of OPCs or containing OPCs that fail to differentiate and remyelinate suggests that ageing and disease duration may be a limiting factor for remyelination in multiple sclerosis (Chang, et al., 2002) (de la Fuente, et al., 2020). This hypothesis is further supported by our results. Specifically, in the whole Paris cohort and in the relapsing-remitting group of the MAGNIMS cohort, we showed that patients with a longer disease duration showed a significant reduction in the efficiency of myelin repair. The evidence that we found a significant association between disease duration and remyelination efficiency only in patients with RRMS, suggests that in this group of patients the failure of myelin repair results from the combined effects of physiological and/or induced oligodendroglial senescence and both acute and chronic inflammatory processes, which are more common in this particular phase of the disease (Boyd, et al., 2013).
In line with the literature, our results suggest that the failure of cortical myelin repair might play a key role in the cascade of events leading to irreversible neurodegeneration (Franklin, et al., 2012) (Ricigliano, et al., 2022) (Tonietto, et al., 2022). In the MAGNIMS cohort, we found that both a failure in cortical remyelination and an increase in the amount of cortical dynamic demyelination were associated with a greater GM atrophy at one year, independently of the amount of demyelination at baseline, age and disease duration (Figure 12). Interestingly, in a recent study of our team we have further shown that the extent of remyelination in periventricular

lesions was significantly associated with cortical thickness and normalized thalamic volume, whereas remyelination in subcortical white matter was not, despite the similar lesional volume of these two regions (Tonietto, et al., 2022). Taken together, these results suggest that cortical atrophy might result from a combination of both persistent demyelination (in particular at the periventricular and cortical level) and failed remyelination. A possible hypothesis to explain these results is that a persistent demyelinating and inflammatory environment in periventricular and subpial lesions, together with a lack of compensatory myelin repair, can drive axonal injury and neuronal loss (Frischer, et al., 2009) (Barnett & Prineas, 2004). In this context, our in vivo data confirm the available evidence from experimental studies, which indicate that remyelination has a key protective role for the axons, preventing both anterograde and retrograde degeneration (Mei, et al., 2016) .

The hypothesis of myelin repair playing a key role in preventing neurodegeneration, could also be supported by our clinical results. In the Paris cohort, we demonstrated that clinical disability in MS, as measured by both EDSS and MSSS, is best explained by the combination of the indices of cortical and WM lesion remyelination. In line with literature, this result may suggest that the failure of myelin repair might play a key role in the cascade of events leading to irreversible neuronal damage and, ultimately, to the accrual of clinical disability, at least in the relapsing-remitting phase of the disease (Franklin, et al., 2012) (Ricigliano, et al., 2022). This result was further confirmed in the MAGNIMS cohort in which, in addition, we found that cortical remyelination has a major clinical relevance in all forms of MS, including the progressive one, but only in patients with a limited extent of cortical demyelination and a short disease duration. Our results suggest that while the process of cortical remyelination can be found at any stage of the disease, including the most advanced ones, its clinical impact in preventing the progressive accrual of clinical disability is limited by a long disease duration and an excessive extent of the demyelinating damage at the cortical level. In other terms, in the first years of disease onset, when the extent of demyelinating damage at the cortical level remains limited, the process of cortical myelin repair is extremely effective in compensating myelin loss, protecting neurons from neurodegeneration and, as a result, in preventing the accumulation of clinical disability in the long-term, regardless of the disease form. However, beyond the first stages of the disease, when the cortex is damaged by an excessive extent of demyelination, the myelin repair process is no longer able to ensure an effective protective role on the axons, which then degenerate over the years leading to the irreversible accumulation of clinical disability.

Our findings suggest that, in order to maximize the probability to prevent long-term clinical progression, people with MS should be offered as early as possible in the course of the disease a combined treatment with DMTs, to reduce the accumulation of demyelination over time, and effective promyelinating therapies, to promote myelin repair both in the white and in the grey matter. The combined effect of these treatments at the earlies stages of MS may have a transformative impact on the evolution of the disease in the long-term. Another relevant implication of our results is that the use of MTR-based individual profiles of cortical myelin repair should be considered in future clinical trials of novel promyelinating therapies as a means of patient stratification and outcome measure, to maximize the probability to find effective treatments to enhance myelin repair in the near future.

Chapter 4.3 - Limits of the studies

Although the study presented in chapter 3.1 has been conducted on a very limited number of patients, which could potentially impact the generalizability of the results, and using MTR, a technique which is not completely specific for myelin (Moccia, et al., 2020) we are confident that cortical myelin repair has a critical role in determining patient disability over time, as confirmed in the much larger MAGNIMS cohort.

Nevertheless, the interpretation of our results still may be challenged by the lack of information about the chronological age of lesions at study entry, by the suboptimal resolution of MTR, particularly when employed to investigate cortical regions, and by the lack of microstructural information which could have helped to better quantify the specificity of the MTR for cortical myelin content, only hypothesised in our studies. As a result, some of the observed heterogeneity in the potential of cortical remyelination might be due to differences in the individual extent of acute demyelination, which is known to be associated with an effective process of myelin repair, to the resolution of inflammation over time, and/or to misclassification errors owing to methodological limitations.

Moreover, in both the Paris and MAGNIMS cohorts, we did not have the opportunity to quantify the extent and localisation of focal cortical lesions detected with appropriate sequences, which has been demonstrated to have a major role in determining disability in MS (Favaretto, et al., 2016) (Calabrese, et al., 2013) (Forslin, et al., 2018), nor were we able to assess the extent and the impact of partial remyelination, which is a common finding in histopathological studies (Chang, et al., 2012).

Of note, our methods to measure remyelination has not been validated against pathology and it is possible that technical variability affects longitudinal measures. However, the reproducibility of the results obtained with distinct imaging metrics (Tonietto, et al., 2022), in multiple-center data (MAGNIMS cohort) together with the limited amount of misclassification errors obtained when generating the maps of myelin content changes (MAGNIMS cohort), support the robustness of our findings.

Our study did not include imaging techniques sensitive to chronic inflammation, and we did not investigate CSF samples for the presence of CSF-derived inflammatory mediators, hampering an explanation of the failure of cortical myelin repair at the molecular level. Finally, our investigation of the relationship between repair failure and neurodegeneration would have been significantly improved by the analysis of other biomarkers of neurodegeneration such as the blood neurofilament levels (Disanto, et al., 2017), that were not available in any of the cohort we studied.

Chapter 5 – Conclusions and Perspectives

In this final chapter, the results previously discussed will be summarized, and the answers to the two key questions proposed in Chapter 2 will be provided. Finally, future perspectives that are indirectly suggested by the results reported in this PhD project will be presented.

1)Is the in-vivo imaging identification of individual profiles of cortical myelin loss and repair in patients with MS possible?

Despite some limitations, mostly linked to the partial volume effect and to the MRI spatial resolution, we provided a new method able to measure patient-specific profiles of cortical myelin content changes.

In the current scenario, in which several resonance techniques have been proposed in the analysis of myelin content, none globally succeeds in achieving an optimal specificity for myelin. Nevertheless, among all, MTR, which was the sequence available for my retrospective and longitudinal studies, have proven to be the most sensitive and specific in detecting minimal changes in both myelin loss and repair (Mancini, et al., 2020), and can be considered a clinically feasible quantitative MRI technique (Chen, et al., 2013). In this PhD project I showed that not only MTR seems to be an excellent technique to explore myelin content but also that the single patients profiles are clinically relevant.

As a matter of fact, it is noteworthy that although our method has not been validated against histopathology, in the present thesis we replicate the results in two clinically different populations. In addition, the same results obtained by means of [^{11}C]-PiB PET, a sensitive and specific technique, were replicated through the use of MTR (Tonietto, et al., 2022). These data together confirm the applicability of MTR in the evaluation of myelin loss and repair at the cortical level.

2) Is the process of cortical remyelination in MS effective in preventing the progression of cortical atrophy and clinical disability over time?

To answer this question, I first focused on the Paris cohort hat I study for the purpose of defining the method. In this population, consisting only of patients with a relapsing-remitting course of the disease and with a particularly high acute inflammatory activity.

The initial results obtained, which subsequently prompted us to further investigate the analysis in a larger and more heterogeneous population (MAGNIMS cohort), showed a relevant clinical role of the cortical remyelination process in which its failure is associated with greater clinical disability and cortical GM atrophy.

In the Paris cohort, as a consequence of the limitations related to the limited heterogeneity of the population, it was not possible to explore the various factors that can influence the efficiency of the myelin repair process. Conversely, in the MAGNIMS, we confirmed on the one hand the extreme variability of the remyelination process at the cortical level, as already demonstrated by the few available histopathological studies, and subsequently confirmed its clinical potential by showing that in the early stages of disease and in patients with a limited extent of cortical demyelination, an increase in remyelination potential is associated with a better clinical outcome at five years.

Moreover, the analysis of the MAGNIMS cohort has revealed that cortical remyelination is able to protect against the risk of short-term cortical atrophy developing over one year, independently of disease duration and MS clinical form, suggesting a key role of cortical remyelination in preventing neurodegeneration.

Future perspectives

Although the results presented in this manuscript have significantly expanded the knowledge about the process of myelin repair at the cortical level, many questions remain unanswered.

The evidence of an acute and chronic inflammatory process at the cortical level compels us to continue our exploration in order to understand the link between cortical microglial activation and the processes of myelin loss and repair. In a study that I am developing, as a continuation of my Ph.D., I am trying to explore cortical microglial activation through the use of the [^{18}F]-TSPO PET tracer, which, as demonstrated for white matter, is sensitive and sufficiently specific for chronic microglia activation.

This project aims to answer two main questions:

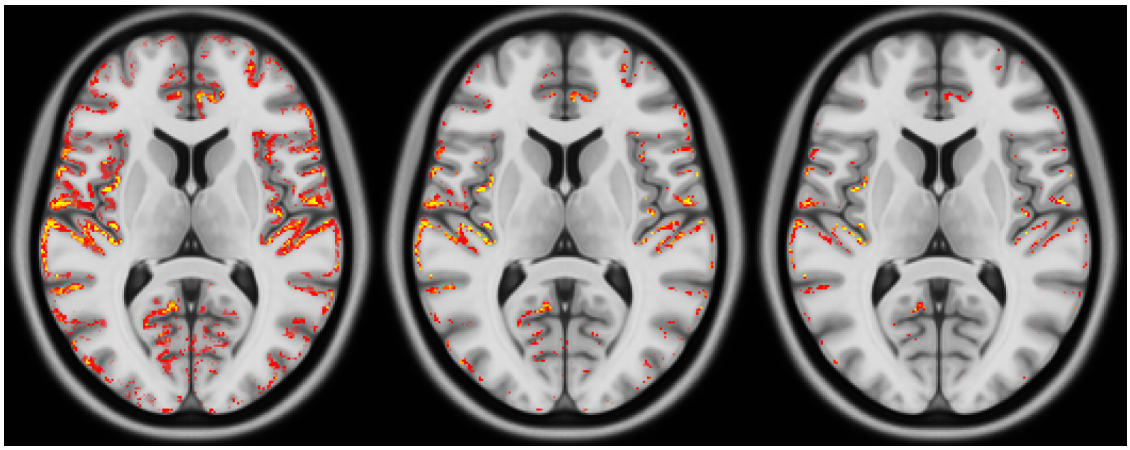
1) Is there a link between microglial activation and cortical remyelination?

2) How does the synergy of the two processes affect physical and cognitive disability and the likelihood of cortical atrophy?

In this study, forty MS patients and nineteen healthy controls have been included, who underwent a neuropsychological assessment and a full MRI protocol at 3T including MTR at baseline and at 12 months. All patients and healthy controls underwent also a [^{18}F]-TSPO PET imaging at baseline which allows to assess microglia activation. MT images will be compared between patients and controls to generate cortical maps of baseline demyelination and dynamic demyelination/remyelination as previously described. [^{18}F]-TSPO PET images will be process in order to define cortical maps of microglia activation, after a preprocessing aimed at re-defining a reference region different from the cortex. The analysis of these data is currently ongoing, and the final results are expected in early 2023.

Preliminary results

- Voxel classification using Z-score, normalized for each cortical region (Desikan-killiany atlas) and creation of probability maps of cortical inflammation

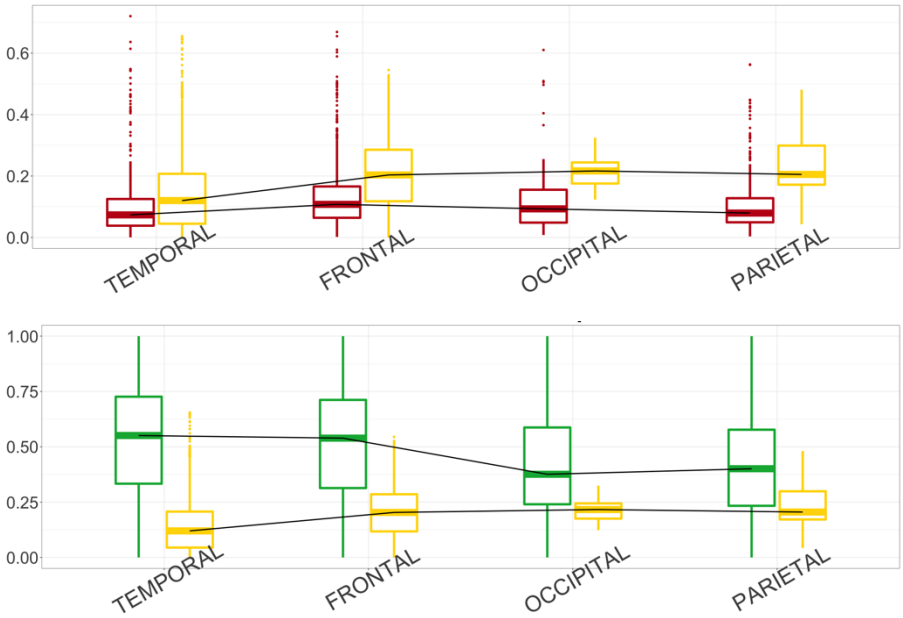


Cortical microglia activation in 25% of patients

Cortical microglia activation in 50% of patients

Cortical microglia activation in 75% of patients

- Trends of association for each cortical lobe (in yellow the % of voxels classified as chronically inflamed, in red the amount of cortical demyelination at baseline, in green the amount of cortical remyelination)



Chapter 6 – List of publications associated with this PhD thesis

1. Cortical remyelination predicts cortical atrophy and clinical progression in early multiple sclerosis.

Lazzarotto Andrea; Hamzaoui Mariem; Tonietto Matteo; Ricigliano Vito Antonio Gerardo; Boffa Giacomo; Khalil Michael; Pirpamer Lukas; Ropele Stefan; Enzinger Christian; Battaglini Marco; Stromillo Maria Laura; De Stefano Nicola; Rocca Maria Assunta; Gallo Paolo; Gasperini Claudio; Stankoff Bruno; Bodini Benedetta for the Magnims Study Group.

Paper under preparation

2. Clinically relevant profiles of myelin content changes in patients with multiple sclerosis: a multimodal and multicompartment imaging study.

Andrea Lazzarotto, Matteo Tonietto, Emilie Poirion, Marco Battaglini, Raffaele Palladino, Charline Benoit, Vito AG Ricigliano, Elisabeth Maillart, Nicola De Stefano, Bruno Stankoff* and Benedetta Bodini*.

Mult Scler 2022 Oct;28(12):1881-1890. doi: 10.1177/13524585221096975

3. Periventricular remyelination failure in multiple sclerosis: a substrate for neurodegeneration.

Matteo Tonietto, Emilie Poirion, Andrea Lazzarotto, Vito Ricigliano, Caroline Papeix, Michel Bottlaender, Benedetta Bodini, Bruno Stankoff.

Brain 2022 Sep 13;awac334. doi: 10.1093/brain/awac334

4. Spontaneous remyelination in lesions protects the integrity of surrounding tissues over time in multiple sclerosis.

Ricigliano Vito Antonio Gerardo, Tonietto Matteo, Hamzaoui Mariem, Poirion Émilie, Lazzarotto Andrea, Bottlaender M, Gervais P, Maillart Elisabeth, Stankoff Bruno, Bodini Benedetta.

Eur J Neurol. 2022 Jun;29(6):1719-1729. doi: 10.1111/ene.15285

5. Increase in neuronal local connectivity following cortical demyelination prevents cognitive impairment in multiple sclerosis

Boffa Giacomo, Hamzaoui Mariem, Lazzarotto Andrea, Ricigliano Vito Antonio, Dubessy Anne Laure, Volkow N, Shokri-Kojori E, Inglese Matilde, Stankoff Bruno, Bodini Benedetta.

The study will be presented at ECTRIMS 2022, and the paper is in preparation.

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