Multimodal imaging assessment of neurodegeneration and neuroplasticity in multiple sclerosis

Eleonora Tavazzi, MD
PhD thesis, 2022

Acknowledgments

First I would like to thank professor Pomeroy and professor Brook, for their kind support, their precious advices and their guidance through these past months. We never met in person, but our meetings ended up being a nice monthly occasion to confront and share thoughts and opinions , on science but not only. I really hope we will be able to share a glass of good wine too, sooner or later.

I would like to deeply thank professor Zivadinov, that I had the luck to meet in 2004 and to keep in touch with until the present days. He has always been a continuous source of intellectual stimuli, a mentor and a friend. It has been a true pleasure to work with him, and I hope we will be able to keep this fruitful and enjoyable collaboration alive for the years to come.

I will always be grateful to professor Bastianello, who sponsored my first “USA adventure”, gave me the opportunity to grow professionally and always supported my initiatives, including this PhD. He defines himself my “pettinabambole mentor”, and I will always thank him for that.

I also want to thank dr. Bergamaschi, who introduced me to the “Multiple Sclerosis world”, taught me most of the things that I know, has always been supportive and soon became a friend, much more than a colleague. I recently came back to his MS Center, and thanks to him it felt like coming back home.

I thank dr.Rovaris, that offered me the precious opportunity to work in his Department and introduced me to the field of Neurorehabilitation. He was always welcoming, supportive, and gave me the possibility to grow up professionally by sharing his great knowledge and methodological rigor. Our professional collaboration lasted less than what we would have liked, but left both of us with the desire to find new ways to work together and share ideas and experiences.

I thank my dad and my dear mum from the bottom of my heart. With their example in mind, I keep putting all my effort in everything I do, trying to do my best as they did and taught me to do. I know where to go only because I have always known that the safest harbor was where I came from, and I could go back to, anytime I needed or wanted to.

I thank my brother Guido and my sister Paola, the best siblings and friends of the entire world. I could have not gone through my life with the same confidence and ability to enjoy it, if I did not have the two of you on my side. One of the certainties of my life, that was sadly confirmed in the recent past, is that if the three of us are together, we can face everything without falling apart. I thank also Ludovica, my sister-in-law. Only biological reasons prevent me from calling her “sister”, as that is what she has been since she came into our family.

I thank my husband, Niels, the other half of the apple that not many persons are lucky enough to find. He is the only one that could have the patience to handle me, through good and bad times, without ever losing his kindness and his caring and loving attitude. We were destined to each other, and to be able to walk through life holding each other's hands has been the most beautiful experience.

I finally thank my three adorable kids, Cecilia, Mattia and Lorenzo and the my little and cutest niece, Mariasole. I once read that to have kids is to decide forever to have your heart go walking around outside your body. Never read anything more true. Hopefully for many years all these pieces of my heart will walk at a very short physical distance from me, and I will be able to enjoy the wonder that they are and the neverending happiness that they bring to my life.

Table of contents

List of abbreviations

ARR	Annualized relapse rate
BBB	Blood-brain-barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
DALYS	Disability-adjusted life years
DGM	Deep grey matter
DMT	Disease modifying treatments
DP	Disease-progressed
DSS	Disability status scale
DTI	Diffusion tensor imaging
DWI	Diffusion weighted imaging
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
FA	Fractional anisotropy
FMRI	Functional magnetic resonance imaging
FTY	Fingolimod
GA	Glatiramer-acetate
GCIP	Ganglion cell and inner plexiform layer thickness
GM	Grey matter
HC	Healthy controls
IFN	Interferon-ß
MD	Mean diffusivity
MOGAD	Myelin oligodendrocyte glycoprotein antibody associated disorders
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSFC	Multiple sclerosis functional composite scale
MSSS	Multiple sclerosis severity score
MTI	Magnetization transfer imaging
MTR	Magnetization transfer ratio

NAWM	Normal appearing white matter
NMOSD	Neuromyelitis optica spectrum disorders
NFL	Neurofilament light chain
NTZ	Natalizumab
OB	Oligoclonal bands
OCT	Optical coherence tomograpgy
ON	Optic neuritis
PML	Progressive multifocal leukoencephalopathy
PMS	Progressive multiple sclerosis
PPMS	Primary progressive multiple sclerosis
PRNFLT	Peripapillary retinal nerve fiber layer thickness
PUFA	Polyunsaturated fatty acids
PWMS	Persons with multiple sclerosis
QSM	Quantitative susceptibility mapping
RCT	Randomized controlled trial
RIS	Radiologically isolated syndrome
RRMS	Relapsing remitting multiple sclerosis
RS-FMRI	Resting state functional magnetic resonance imaging
SNFL	Serum neurofilament light chain
SPMS	Secondary progressive multiple sclerosis
T2-LV	T2-lesion volume
WM	White matter

Purpose of the thesis

Multiple sclerosis (MS) is the most common non-traumatic cause of disability in young adults. Neurodegeneration, closely intertwined with neuroinflammation, is primarily responsible for disease progression, through a process of gradual accrual of brain tissue damage and progressively increasing clinical impairment. A better understanding of the pathogenetic mechanisms underlying MS progression would facilitate the identification of novel pharmacological and non-pharmacological therapies capable of counteracting the detrimental effects of the disease. This would ultimately lead to a reduction of disability accrual, with several relevant consequences at an individual level (physical, psychological, social and work-related), as well as at the global level, in terms of disease-related costs. Neuroimaging techniques have a relevant role in this context, as they allow for an in vivo, non-invasive monitoring of MS evolution. With this general background, the main aim of the current project is the identification of novel MRI markers able to broaden our knowledge on the mechanisms underlying disease progression.

Abstract

Background The advent of advanced magnetic resonance imaging (MRI) techniques has allowed to study multiple sclerosis (MS) evolution over time, providing qualitative information and quantitative measures related to tissue damage. However some relevant questions remain unanswered, mainly concerning a better definition of structural and functional features of disease progression as well as the identification of causal factors for disease evolution over time

Aims to characterize different aspects of disease progression, by applying a multimodal imaging approach.

Methods Together with traditional measures of lesion volumes, such as T1-weighted and T2-weighted lesion loads, and of global and regional brain volumes, we applied: quantitative susceptibility mapping (QSM) to quantify iron; diffusion tensor imaging (DTI) to analyze microstructural tissue properties; functional MRI (fMRI) to analyze activity pattern in response to a motor task; resting state fMRI to analyze functional connectivity within regions of interest; optical coherence tomography (OCT) to evaluate the usefulness of an alternative imaging technique in the study of neuroaxonal damage

Results We showed that disease progression is related to iron dyshomeostasis in deep grey matter structures. We confirmed the usefulness of a novel MRI marker such as atrophied T2-LV, reflecting both lesion accrual and simultaneously occurring irreversible tissue loss. Moreover, we confirmed the role of sNfL and OCT measures as markers reflective of neuroaxonal degeneration. Finally, we analyzed the effect of motor rehabilitation on clinical and MRI markers, supporting its usefulness as a non-pharmacological treatment able to impact on disability.

Discussion/Conclusions The findings here reported highlight that MS disease progression cannot be properly characterized using conventional radiological imaging, or even a single advanced neuroimaging modality. Only the combination of structural and functional MRI techniques investigating complementary aspects of MS-related pathogenetic mechanisms can lead to a better understanding of disease progression.

Papers included in the thesis

1. Zivadinov R, **Tavazzi E,** et al. *Brain Iron at Quantitative MRI Is Associated with Disability in Multiple Sclerosis.* Radiology. 2018 Nov;289(2):487-496. In this study, we applied quantitative susceptibility mapping (QSM) both in regions of interest, such as thalamus and basal ganglia (BG), and also in a voxel-wise analysis, aiming at better characterizing deep grey matter (DGM) structures susceptibility in a large MS sample.[1] No. of citations: 44
   The candidate contributed to design of the investigation, and performed entirely the analysis of the outcome, as well as prepared the work for publication.

2. Tavazzi E, et al. *A multimodal approach to assess the validity of atrophied T2-lesion volume as an MRI marker of disease progression in multiple sclerosis*. J Neurol. 2019 Nov 25. The current study aimed at analyzing the association between atrophied T2LV and other imaging and clinical markers of MS progression, and compare all the markers between MS patients showing disease progression (DP) at follow-up and stable MS patients.[2] No of citations: 4
   The candidate contributed to design of the investigation, and performed entirely the analysis of the outcome, as well as prepared the work for publication.
3. Tavazzi E, et al. *Serum neurofilament light chain and optical coherence tomography measures in MS: A longitudinal study*. Neurol Neuroimmunol Neuroinflamm. 2020 May 18;7(4):e737. The specific aim of the study was to analyze the association between OCT measures and a serum biomarker of axonal degeneration, the serum neurofilament light chain (sNfL), comparing both MS patients with age- and sex-matched healthy controls (HC), and also within the MS group, relapsing-remitting MS (RRMS) versus progressive MS (PMS).[3] No of citations: 10

The candidate contributed to design of the investigation, and performed entirely the analysis of the outcome, as well as prepared the work for publication.

4. Tavazzi E, et al. *Effects of motor rehabilitation on mobility and brain plasticity in multiple sclerosis: a structural and functional MRI study*. J Neurol. 2018 Jun;265(6):1393-1401. With this study we aimed at evaluating the effect of an intensive motor rehabilitation on functional and structural brain rearrangements.[4] No of citations: 33
   The candidate performed entirely the design of the investigation, conducted the research and performed the analysis of the outcome, as well as prepared the work for publication.

Reviews reported in the Appendix

5. Tavazzi E, et al. *MRI biomarkers of disease progression and conversion to secondary-progressive multiple sclerosis*. Expert Rev Neurother. 2020 Aug;20(8):821-834. This review encompasses a description of histopathological, clinical and MRI features of MS progression and conversion to the progressive disease stage.[5]
6. Bergsland N*, Tavazzi E,* *Targeting Iron Dyshomeostasis for Treatment of Neurodegenerative Disorders.* CNS Drugs. 2019 Nov;33(11):1073-1086. This review offers a brief overview of the role of iron within the brain in physiological conditions as well as some pathogenic hypotheses on the potential contribution of iron dyshomeostasis in the most common neurodegenerative disorders.[6]
   * shared first authorship

Chapter 1. Introduction

Brief historical overview of multiple sclerosis

The first clinical description of multiple sclerosis (MS) might be the case of a young Dutch girl that developed, in 1395 what was likely to be an optic neuritis, followed by gradually accumulating motor disability.[7] However, the first systematic description of this new nosological entity is from 1868 when Professor Charcot reported about "sclerose en plaques", describing symptoms that he considered typical of the disease "intention tremor, nystagmus, and scanning speech". [8]

The 20th century has marked several milestones in the history of MS. The most important discoveries concern the possible role of genetics in the etiopathogenesis of the disease from F. Curtius in 1933,[9] the presence of oligoclonal bands (OB) in 1940.[10] Moreover, the advent of magnetic resonance imaging (MRI) in the second half of the century has revolutionized the approach to several diseases, including MS, allowing to study in vivo what was possible to analyze only from an histological point of view.

The first diagnostic criteria were defined by Schumacher and colleagues in 1965 and based only on clinical features, respecting the rules of dissemination in space and time. To be diagnosed as MS, a patient needed to show two separate attacks in time, involving different functional systems.[11] The following version of the diagnostic criteria was formulated by Poser in 1983, introducing the distinction between "clinically definite MS" and "probable MS", as well as including paraclinical evidence to confirm the spatio-temporal dissemination, by means of the presence of OB exclusively in the cerebrospinal fluid (CSF).[12] The introduction of MRI as a tool to diagnose MS and monitor the disease evolution led first to defining diagnostic MRI criteria capable of predicting the conversion from a first episode of suspected MS to clinically definite MS, [13-15] and then, to further revise the general diagnostic criteria.[16] Indeed, MRI can track the evolution of MS plaques - and the number and location of these are partially related to degree of disability. In particular, conventional MR T2-weighted images allow to identify lesions demyelinating whereas T1-weighted sequences allow to visualize chronic lesions as well as active lesions, if using Gadolinium contrast

enhancement. The following three revisions, including the most recent one published by McDonald in 2017, were motivated by the need of reaching a definite diagnosis as early as possible after disease onset.[17-19] In the meantime, a substantial change in the natural disease course occurred, due to the positive results of the first randomized controlled trial (RCT) testing a drug in MS. Interferon ß-1b was approved in 1993 by the food and drug administration (FDA) as the first pharmacological compound effectively reducing on relapse rate.[20] Since then, several other treatments have become available and the approach of clinical neurologists has changed dramatically after several studies showed that treating the disease as early as possible in the course of the disease favorably improves the long-term prognosis. [21,22] This has focused attention on developing biomarkers that can identify patients with MS, or at risk of developing MS, at the earliest point.

As of today, there are more than ten available pharmacological therapies for the relapsing-remitting form of MS (RRMS) and several ongoing RCTs of new compounds. Different mechanisms of action and administration route allow for the tailoring of therapeutic choices to the level of disability, inflammatory activity and needs of each individual patient. Furthermore, for the first time, two drugs have shown some efficacy in slowing down disease evolution in progressive forms of MS (ocrelizumab and siponimod).

Even though a specific biomarker for the disease has not been found yet, MS is now considered a multifactorial disease in which the immune-mediated reaction results from the combination of genetic predisposition and several identified environmental factors. The availability of advanced MRI techniques and sophisticated laboratory tools will allow expansion of knowledge of the mechanisms of the disease, possibly identifying more efficacious treatments for both the neuroinflammatory and neurodegenerative components of the disease.

Disease sub-types and measures of clinical disability

There are three main disease sub-types in MS: relapsing-remitting (RRMS) , primary progressive (PPMS) and secondary progressive (SPMS).[23]

RRMS is characterized, as suggested by its definition, by acute symptomatic phases (i.e., relapses) followed by partial or complete recovery (i.e., remission) and subsequent periods of a stable disease course. It is the most common disease form, accounting for up to 85% of patients. In the natural disease history of MS, after 20-25 years from onset, the majority of RRMS convert to SPMS, a phase characterized by apparently reduced inflammatory activity paired with a gradual, irreversible disability accrual.

Around 10% of people present with PPMS, in which there is a gradual onset of symptoms followed by a slow and irreversible gain of disability. There is still debate about the existence of an entity called "benign MS", that has different definitions generally focused on the presence of low disability after a long disease duration. Common definitions include, for example, a low disability score on the Expanded Disability Status Scale (EDSS) (i.e.EDSS < 3.0) after 10,15 or 20 years of disease duration.[24] More recently, it has been suggested that benign MS should be defined when respecting the following criteria: EDSS ≤1.0, absence of disability, and the ability to work after 15 years of disease duration.[25] The more widespread use of advanced MRI techniques and availability of large scale longitudinal cohort studies following patients over several decades will allow to clarify the real existence of this form, and possibly the pathogenetic features favoring such a favorable disease course. [25] The latest version of diagnostic criteria allowed to include in the spectrum of MS-related forms other two entities: clinically isolated syndrome (CIS) and radiologically isolated syndrome (RIS). The former is characterized by the presence of a single clinical episode, associated with lesions at MRI with typical features. The latter diagnosis is

formulated when MRI lesions typical for MS are detected, in absence of any suggestive symptoms.[26]

As mentioned earlier, the first symptoms described as pathognomonic for all forms of MS, the so-called "Charcot's triad" were intention tremor, nystagmus, and scanning speech.[8]
However, as the tissue damage, either focal or widespread, can potentially affect the whole CNS, the disease can present with a variety of symptoms belonging to eight defined functional systems, classified in visual, pyramidal, cerebellar, brainstem, sensory, bower/bladder, cerebral (mentation), other.[27] The need to quantify disability in a reproducible fashion led to the identification of the so-called disability status scale (DSS), [28] subsequently modified in the Expanded Disability Status Scale.[29] After more than 30 years and despite several pitfalls, EDSS remains the most common scale applied both in clinical practice and in the context of RCTs.
EDSS ranges from "0" (i.e. normal neurological examination) to "10.0" (i.e. death due to MS) with 0.5 increments. The global score derives from the non-linear sum of the scores of each single functional system that can range from "0" (no signs/symptoms) to "5" or "6" (marked disability). However, this rule is mainly applicable to scores from 0 to 4.5 whereas patients are attributed a score ranging from 5.0 to 9.5 mainly on the level of impairment of their motor function, thus requiring a walking aid. The fact that EDSS is heavily weighted towards the motor functions is also one of the main criticisms, together with the modest inter-rater reproducibility.[30]
However, attempts to identify more reliable scales have failed so far, either because they share the same limits of EDSS or because they are more focused on one specific aspect of disability. [31]
The multiple functional composite scale (MSFC), a three dimensional scale investigating leg function/hand function/cognition through specific, validated tests, has gained some interest as presented an acceptable validity and sensitivity to clinical changes over a short timespan (1-2 years).[32] The need for specialized equipment (i.e. the 9-hole-peg test apparatus, an audio CD with a

CD reader) and assessment time, around 20-30 mins, makes the scale more suitable for RCTs than clinical practice.

Multiple sclerosis severity score (MSSS) combines EDSS with the distribution of disability among patients with comparable disease duration, trying to assess disease severity.[33] Although it showed a good reliability in predicting disability progression at a group level, the unpredictable disease course of a single patient, probably influenced by several factors, limits its use at an individual level.

Epidemiology of MS

Multiple sclerosis is the first most common neurological disease in young people as well as the most common non-traumatic cause of disability in youngsters.[34,35]

A recent edition of the "Atlas of MS" showed that the total number of persons with MS (pwMS) is approximately 2.8 million across the globe, versus 2.3 million reported in 2013. (www.atlasofms.org).

Several epidemiological studies have described an increasing trend of the incidence of MS in the last century, although some others reported opposite results, showing a stable or even decreased incidence over time .[36-40] A recent review has summarized the results of the most relevant epidemiological studies in this area, confirming an increase of the overall incidence weighted by the square root of number of patients per data point between 1950-2000, with a plateau after that.[41]

There are few possible explanations for this trend. On one hand, the general awareness towards health-related issues has markedly increased, especially in developing countries, together with the availability of health services, leading to an increase of diagnoses, and not necessarily of real prevalence. For example, the number of neurologists in the USA showed a more than six-fold increase between 1970 and 2010.[42]

The source of data analyzed in the studies might have played a role as well, as quite often the results were based on administrative claims databases or insurance data that might have been collected and analyzed in different ways across time, leading to varying results.[41]

Another relevant change over time concerns the diagnostic criteria used to ascertain a case of MS. With respect to the first version, the so-called Schumacher criteria, published in 1965, that allowed a diagnosis based on the two main clinical features typical for MS-dissemination in time and space, the following versions incorporated the presence of OBs and evoked potentials and, finally, MRI.[12,16] In particular, the introduction of MRI has dramatically changed the sensitivity of the criteria, because the presence of brain or spine lesions at different stages has been considered a valid alternative to clinical attacks for the demonstration of spatio-temporal dissemination. The most important consequence has been the increase in detected MRI lesions compatible with the diagnosis but no signs of dissemination in time, which earlier might have been classified as CIS. On the other hand, the abovementioned trend might reflect a real increase of MS incidence due to the changes in lifestyle and exposure to different risk factors. The following paragraphs will describe the most commonly reported factors affecting susceptibility and/or disease course related to MS.

Sex

Females are characterized by a higher risk of developing MS, as well as other autoimmune diseases, together with a better long-term prognosis with respect to their male counterparts. Recent data showed a progressive increase of the MS incidence in women, even though with a wide variability among different areas throughout the world. Commonly, the reported sex ratio of women: men was 2:1, reaching 3 to 4:1 in several regions,[43-45] although lack of change over time has also been reported.[46]

Again, this might be an apparent effect reflecting improved access to health services for women, at a socio-economic level, but it can also be causally associated with the dramatic societal change in women's lives over the last century. Recently, women have been more actively involved in

professional activities outside the home environment, with a change in the nutritional habits as well as potentially higher exposure to air pollutants. Another consequence of the higher employment rate for women is the downward trend in pregnancies, which are protective against inflammatory episodes (e.g., relapses)[47]. Whether this might be another factor contributing to the increased incidence of MS in women needs to be clarified, but a change in the hormonal setting might definitely impact on the sex-related epidemiology of the disease.

Notably, the COVID-pandemic has had a dramatic impact on women in the workplace. Specifically, women were more likely than men to be unemployed, while those still working had greater tendency to work from home. [48] Whether these changes will be permanent and possibly associated with a reduced incidence of MS in women, will be the subject of future studies.

Latitude and sun exposure

Worldwide epidemiological studies have consistently shown a strong latitude gradient, with the highest prevalence towards the north northern and south southern parts of the hemispheres, along with a reduced trend of incidence, prevalence and mortality around the Equator.[49-52] This has been attributed to sun exposure and, by inference, to vitamin D levels[53,54] Supporting evidence comes from both animal, clinical and MRI studies reporting an association between vitamin D levels, mainly early in life, and the risk of developing MS, presenting higher relapse rate and/or showing signs of MRI activity.[55] On the other hand, implementation of vitamin D supplementation did not show any conclusive benefit on clinical outcomes such as relapse rates, as well as conflicting results on MRI parameters such as the number of active lesions, identified using gadolinium contrast enhancement, or new T2 lesions.[55] Future larger randomized studies are needed to clarify the potential role of vitamin D supplementation on clinical and MRI outcomes in MS.

A possible confounding element when analyzing environmental factors influencing MS prevalence concerns migrants. With respect to latitude, in particular, internal and external migration from areas characterized by different prevalences might affect results of epidemiological studies. Recently, Sabel et al. analyzed lifetime residential calendars in New Zealand, building lifetime latitudinal

gradients.[56] Interestingly, when taking into account not only the patients location at the time of diagnosis but the location at birth and possible subsequent migrations, the Authors described a strong geographical prevalence gradient at birth that gradually declined after age 12, mainly driven by females with a relapsing remitting disease course. This underlines the importance of considering exposure to environmental risk factors as early as in utero and at birth.

Another aspect that needs to be taken into account concerns the fact that different latitudes are often characterized by several other relevant differences at cultural, social and genetic levels. Therefore, sun exposure is only one of many factors that can contribute to the latitude gradient.

Smoking

MS can be listed among the several diseases causally associated with smoking habits, both in terms of susceptibility and disease progression, in some cases with a demonstrated dose-dependent effect.[57-59] There is experimental evidence for a relevant role of the lungs in the pathogenesis of MS that can explain the relevance of smoking habits in MS. The bronchus-associated lymphoid tissue represents a niche in which T-lymphocytes acquire the migratory abilities that will facilitate their passage into the central nervous system (CNS) through the blood-brain-barrier (BBB).[60] Lung tissue of smokers is characterized by an inflammatory environment, and animal studies described increased macrophage activation, microglial cells with cytotoxic activity,[61] which favor the activation of autoaggressive T-lymphocytes.

Several studies showed also an association between smoking and worse clinical outcomes, in terms of both faster disability gaining and a more rapid evolution into the progressive phase of the disease,[62,63] as well as on MRI markers of neuroinflammation and neurodegeneration.[64] Moreover, smoking cessation is associated with a slowing of motor disability progression, further confirming the detrimental effects of smoking on MS.[65,66]

Air pollutants

An association with air pollution has been noted for many health-related issues, including allergic, oncologic, cardiologic and neurologic diseases. Air pollutants exert detrimental effects on CNS by

increasing the level of proinflammatory cytokines and oxidative stress byproducts at a lung as well as CNS level, promoting neuroinflammation, neurodegeneration and BBB breakdown, which are key features of MS pathogenesis.[67,68]

Air pollutants can be categorized according to the diameters of the particulate matter (PM), and the most commonly studied are PM10, PM2.5 and ultrafine PM. Several studies have investigated the possible causal role of PM10 in MS, concordantly describing an increased prevalence in urban areas characterized by higher levels of PM10.[69-71] Moreover, recent exposure to PM10 levels are associated also with the onset of clinical relapses and appearance of new contrast-enhancing lesions.[72-74] The same positive evidence of an association between the level of air pollutants and MS susceptibility has been detected with respect to PM2.5.[75,76]

Even though results of the aforementioned studies strongly suggest an association between the level of air pollutants and the risk of MS, the relapse rate, disability level, as well as inflammatory MRI markers, some general methodological limitations that might hinder interpretation of the results need to be taken into account. For example, most studies analyzed the level of air pollutants in the living area of the participants, without correcting for the amount of time spent in that specific area on a daily basis; measurements are taken with different devices, rendering direct comparisons between studies problematic. Moreover, several factors like smoking habits, seasonal variation of ultraviolet exposure might play relevant interfering roles. In conclusion, several epidemiological studies have reported an association between air pollution and MS, both in terms of susceptibility and clinical/MRI inflammatory markers, substantiated by experimental evidence of a detrimental role of air pollutants.[77] However, the methodological limitations of these studies, motivate the need for better designed experiments to investigate this crucial topic.[78]

Viruses

While searching for an etiological agent responsible for MS, several infectious agents have been investigated like Epstein-Barr virus (EBV), Bordetella pertussis, measles virus and many others. In particular, EBV has gained considerable attention for several reasons: nearly all pediatric MS

patients are EBV positive, as well as virtually all adult MS patients.[79-82] However, EBV is nearly ubiquitous among the general population, making it difficult to establish a definite causal association exclusively based on epidemiological data. A recent epidemiological study carried out on a wide population of young people on active duty in the US military service, reported a 32-fold increase of developing MS in people infected with EBV, as well as an association between EBV infection and increased levels of serum Neurofilament light chain (sNfL), a biomarker rof neurodegeneration.[83]

Moreover, there is an association between the blood levels of antibodies against EBV and the onset of MS, as well as between antibodies against the EB nuclear antigen (EBNA) and the onset of acute relapses.[82,84] EBV is able to activate several mechanisms to escape active immune surveillance within the infected body, and establishes a lifelong latent infection of B cells.[84] This entails, among other consequences, a progressive, chronic depletion of CD8+ T-lymphocytes that would promote the inflammatory substrate necessary for MS onset, while reducing the ability of T-lymphocytes to limit EBV reactivation.[84] EBV-infected B cells circulate into the bloodstream, and also traffic through the BBB, triggering an inflammatory process both in periphery and within the CNS. Therefore, EBV could potentially play a role both in inducing relapse onset and in appearance of new T2 lesions and contrast-enhancing lesions, as well as maintaining a chronic inflammatory state compartmentalized within the CNS. Indirect evidence for a crucial role of EBV in the disease pathogenesis comes also from the high efficacy of ocrelizumab, a new pharmacological treatment approved for MS that specifically targets anti-CD20 B cells. Future development of pharmacological compounds selective against EBV-infected B cells would help address the outstanding questions regarding the pathogenetic role of EBV in MS.

Diet

Several elements have recently drawn attention towards the potential association between diet quality and MS.[85] First, the discovery that the gut microbiome might be involved in the pathogenesis and progression of several autoimmune disorders, among which MS, [86] has led to the

careful consideration of the potential proinflammatory influence of nutrients. Moreover, several observational studies have reported an association between MS and obesity, especially in childhood.[87-89] The biological explanation of this phenomenon might reside in the fact that elevated body mass index, through increased levels of adipose-derived hormones seems to promote a proinflammatory immune milieu.[90-92] Some preclinical and clinical data suggest that different dietary patterns might be related to the level of inflammatory activity: for example a high dietary intake of saturated fat and/or salt increases the risk of relapse rate,[93] whereas diets rich with produce and whole grains are associated with a lower relapse rate.[94,95] Moreover, some observational and small randomized controlled studies reported a beneficial effect of dietary regimens enriched with polyunsaturated fatty acids (PUFA) on the risk of MS onset,[96-99] likely due to a cytokine-mediated anti-inflammatory effect.[100]

Specific dietary regimens such as the ketogenic diet, fasting regimens, and the Mediterranean diet, have been evaluated in MS patients and animal models with global positive results,[101] both reducing inflammation, favoring recovery and cellular regeneration and favoring neuroprotection.[101,102]

However, most of these results derive from observational studies, or very small studies in which possible confounding factors, limited sample size and other methodological pitfalls may have hampered the interpretation of the data. Indeed, a recent Cochrane review reported no conclusive evidence for dietary supplementation with PUFA, vitamins or antioxidants, but underlines the potential interest of the topic, advocating for larger, well designed randomized studies.[103]

Pathophysiology of MS

Multiple sclerosis (MS) was traditionally considered an inflammatory disease of the central nervous system (CNS) affecting predominantly the white matter (WM) and leading to the formation of focal demyelinating lesions. The advent of conventional and advanced MRI techniques as well as of basic sciences and techniques such as immunohistochemistry radically expanded knowledge of the pathophysiology of the disease. It is now well established that the disease involves both grey matter

(GM) and WM since the early stages, with two main pathogenetic mechanisms responsible for the ongoing tissue damage, namely inflammation and neurodegeneration.

Neuroinflammation-white matter

Focal plaques of active demyelination are such a pathognomonic feature that MS was initially named "sclerosis en plaque" after them. Histopathologically, active lesions are characterized by demyelination, neuroaxonal damage and astrogliosis with inflammatory infiltrates, but distinctive features allow to classify them into four different types.[104] Type I and II are characterized by sharp edges, perivenular location of the lesions, and the presence of T-lymphocytes and macrophages infiltration, together with antibodies and complement antigen deposition in type II lesions. Type III are still characterized by T-lymphocytes and macrophages infiltration, but they have less demarcated edges, are not centered around vessels but spreading into the normal appearing WM and show signs of oligodendrocyte damage and apoptosis with a preferential loss of myelin-antigen-glycoprotein (Type III) among all myelin proteins. Finally, type IV lesions share some features of type I and II, such as the perivenular location, T-lymphocytes and macrophages infiltration and the loss of all myelin proteins, but also signs of oligodendrocyte death, and the absence of remyelination.

Notably, the aforementioned study by Lucchinetti and colleagues revealed inter-patient heterogeneity but intra-patient homogeneity of lesions, suggesting the presence of different pathogenetic mechanisms underlying each lesion type.[104] In particular, type I and II lesions present features similar to the histopathological findings of animal models of autoimmune encephalomyelitis, supporting an autoimmune mediated pathogenesis, whereas the damaged oligodendrocytes of type III and IV lesions suggest a toxic or virus-mediated mechanism. The clinical relevance of different lesion patterns has been recently reported: there is no association between lesion patterns and age or sex; pattern II has repeatedly been reported as the most frequent lesion pattern, and is the most represented in patients with disease duration longer than five years.[105] Patients with pattern III lesions are more likely to have higher disability and were more represented

among autopsy cases of patients died for causes related to MS. Interestingly though, no lesion pattern was associated with a negative outcome or a higher likelihood to convert to a progressive form of disease.

Interestingly, an autoptic study analyzing active demyelinating lesions regardless of the time of appearance reported the same histopathological findings for all the lesions, represented by antibodies and complement deposits associated with macrophage infiltration in areas of active demyelination, but no oligodendrocyte damage or preferential myelin protein loss.[106] This might suggest an initial diversity of pathogenetic mechanisms triggering the appearance of inflammatory demyelinating lesions, ultimately converging into a same dysimmune, self-sustained process of tissue damage.

Remyelination is generally absent or incomplete in MS lesions, as the ability of oligodendrocytes progenitors to differentiate is limited. However, in a small percentage of cases it is possible to observe thinly remyelinated lesions, named "shadow-plaques".[107,108]

The advent of MRI has dramatically expanded our knowledge on the disease, allowing us to detect lesions *in vivo*, and follow their evolution over time. Conventional MRI techniques in the context of the MS clinic as well research refer to standard imaging modalities, such as T1-weighted and T2-weighted sequences. Both types of images are readily capable of detecting damage within the CNS due to their sensitivity to altered water content and molecular water environment. T2-weighted images are typically considered the most sensitive to tissue damage, and are usually applied to identify focal lesions in the context of MS. The administration of a gadolinium-based contrast agent can be utilized to detect areas of acute tissue injury, reflective of an impaired blood brain barrier, due its T1 shortening effects, thus resulting in T1 hyperintensity.[109] Other MRI techniques, such as magnetization transfer imaging (MTI) and susceptibility weighted imaging (SWI), exploit physiochemical properties of the tissue to generate tissue-contrast. A newly forming lesion usually appears as T2-hyperintense, T1 iso-hypointense, as well as hyperintense in T1-weighted images

after contrast injection (the so called contrast enhancing lesions). However, despite their sensitivity to tissue damage, T2-weighted images are highly unspecific. For example, areas of T2 hyperintensity in MS can reflect a myriad different pathological processes, including demyelination, oedema, gliosis, and axonal injury.[110] T1-weighted hypointensity is somewhat more specific and reflects more severe tissue destruction, at least when visualized with spin-echo acquisitions, but are still relatively non-specific to the underlying pathology.[111] Importantly, neither type of acquisition can be used to distinguish between acute vs. old lesions with only a single cross-sectional assessment, although a marked T1 hypointensity reflects scarring typical of chronic lesions, consequently named "black-holes".

All three lesion types, T2-and T1-hyperintense and T1-hypointense, have played an important role in the diagnosis and clinical follow-up of patients with MS. Disappointingly though, even quantitative measures (e.g., lesion count, lesion volume) have shown relatively poor relationships with clinical outcomes, a finding which has been termed the clinico-radiological paradox.[112] For example, some patients may present with a considerably extensive pattern of lesion activity but nevertheless remain relatively free of substantial impairment from the disease while others may have a limited lesion load but end up being severely disabled. A number of studies have aimed to explain these seemingly discrepant findings. It has been shown that lesion location can play a crucial role,[113] which can be intuitively understood by considering the strategic location of a given lesion. A lesion that interrupts the corticospinal tract is likely to have a much stronger impact on clinical disability compared to one in an association fiber tract such as the cingulum. Despite such limitation, quantitative measures of lesion volume and count continue to remain a mainstay in clinical trials of multiple sclerosis drugs, often representing primary outcomes, with the expectation that an efficacious treatment will reduce the appearance of new lesions compared to either placebo or other pharmaceutical compounds that exert a weaker effect on the disease.

In the late disease stages, the number of active demyelinating lesions gradually subside, in favor of chronic-active and chronic lesions. The former, also called smoldering plaques or slowly expanding

lesions, are histologically characterized by an inactive center, with no inflammatory infiltration, and a rim of activated, iron-laden microglia/macrophages with myelin debris. Smoldering plaques are the hallmark of a chronically active inflammation process constantly ongoing within the CNS, typical of late-disease stages.[114,115] Conventional MRI has rarely been applied to detect smoldering lesions and is limited by the need of longitudinal assessment, [114,116] whereas advanced techniques sensitive to iron enable the identification of this type of lesion cross-sectionally, based on the presence of a paramagnetic rim. [117]

However, WM is affected not only focally but also on a global level with a variable degree of diffuse damage, and histopathological evidence of axonal swelling and degeneration, demyelination, microglial activation and inflammatory infiltration.[118] Wallerian degeneration caused by an axonal transection within focal lesions might be partially responsible for the subtle, diffuse damage occurring throughout the so-called normal appearing WM (NAWM)[119-121] The term NAWM refers to the fact that this tissue may appear to be unaffected when viewing conventional imaging sequences. This type of tissue damage is difficult to visualize with conventional MRI techniques, but tissue loss can be quantitatively assessed, as explained in the section on neurodegeneration.

Neuroinflammation-Grey matter

Neuroinflammation within GM is still characterized by demyelination and inflammatory infiltration as well as oligodendrocyte damage, but the degree of inflammation and the amount of microglia/macrophages infiltration are smaller than what is typically found within the WM. [122,123] Focal lesions are detectable also within cortical and deep GM, as well as cerebellar cortex. Cortical lesions are classified according to the site and histopathological type, as follows: type 1, cortico-subcortical and expand both in the GM and WM; type 2 are small, intracortical, with a perivenular location; type 3 are most frequently reported in progressive MS forms, located in the subpial cortical layers and associated with leptomeningeal inflammation; type 4 are purely cortical and extend throughout all the cortical layers.[122-125] Due to anatomical location and histological

composition, cortical lesions have always represented, and partially still represent, a challenge to visualize with MRI. The development of the imaging technique called double inversion recovery has markedly increase the possibility to detect focal lesions within cortical GM, [126] further improved by using ultra-high field MRI. [127] Several studies have shown the correlation between cortical lesions and clinical disability, as well as cognitive impairment in MS, further underlining the role of GM pathology in MS.[128,129]

Neurodegeneration

Unlike the most prominent focal expression of neuroinflammation, neurodegeneration is characterized by a widespread, subtle damage affecting both WM and GM. It is of paramount importance from a clinical point of view as it is the mechanism mainly responsible for the gradual disability accrual typical of late disease stages. The histopathological hallmarks of neurodegeneration are axonal and neuronal damage, associated with oligodendrocyte degeneration. Traditionally the neurodegenerative component was considered as a secondary phase, temporally and causally associated to neuroinflammation, and more specifically to the transition between peripheral inflammatory cells entering the CNS through a disrupted BBB and compartmentalized neuroinflammation driven by soluble factors produced by B lymphocytes organized into follicle-like structures located in the leptomeninges.[130] Recent evidence has showed that neurodegeneration is present from the very beginning, and its role might be more crucial in the disease evolution than the mere consequence of neuroinflammation, to the point that two different pathogenetic mechanisms have been postulated: the “inside-out” vs the “outside-in” models. The latter hypothesizes a first, unknown, peripheral dysimmune trigger that initiates the neuroinflammatory process leading to the entry of T- and B lymphocytes within the CNS, with, as a consequence, inflammatory cells and soluble components spreading within WM and GM, causing focal lesions. The former model, instead, attributes a central role to an initial neurodegenerative component, with neuronal and oligodendrocyte damage, exposing antigens and releasing factors that trigger an inflammatory response. [131,132] Even though the question about what process comes first remains

unanswered, histopathological studies describe the two processes as closely intertwined, with active inflammation and acute axonal injury contemporarily present in lesions, and simultaneously gradually decreasing in late disease stages.[133] Together, chronic inflammation and neuroaxonal damage leads to a progressively increasing tissue loss that correlates with clinical disability. Atrophy, or the progressive loss of CNS tissue that reflects neurodegeneration, can be appreciated using conventional MRI images as well. Notably features of tissue loss within the brain include enlargement of the ventricles as well as widening of the sulci. Traditionally, brain atrophy was assessed using qualitative reads but over the last two decades, a number of quantitative techniques have been proposed in the literature and widely used in the MS field. A far from comprehensive list of examples that rely, at least to some extent, on manual measurements includes third ventricular width[134] and bicaudate ratio.[135] Fully automated techniques have also been developed such as brain parenchymal fraction[136] (a ratio of the total brain tissue to intracranial volume) and normalized brain volume[137] (brain tissue normalized for head size). Importantly, these are inherently cross-sectional measures that essentially provide a "snapshot" of the degree of atrophy, whether it be region specific or at the global level. One of the most widely used longitudinal extensions of brain atrophy measurements is SIENA (Structural Image Evaluation, using Normalisation of Atrophy) technique, which provides a measurement of percent brain volume change from a pair of structural MRIs.[137] Compared to conventional measures of lesion volume, atrophy measures are typically more informative with respect to long-term disability progression.[138-142] Nevertheless, a number of aspects need to be considered. First, tissue atrophy is an end-stage phenomenon, and its assessment does not capture the changes that occur prior to it. Second, there are a number of biological and technical factors that can hamper interpretability, particularly when considered at the individual level. For example, various factors such as hydration status, menstrual cycle, and time of day can affect the precision and accuracy of its measurement.[143] Moreover, changes in scanner platform or sequence typically render atrophy measurements invalid, especially in multicenter settings. Although there has been considerable enthusiasm in incorporating atrophy measurement into the

clinic, as evidenced by FDA approved / CE-marked tools for its assessment, the aforementioned factors remain difficult to overcome, despite ongoing research. In fact, initial excitement concerning the incorporation of atrophy measurement into the so-called no-evidence of disease activity criteria[144] has been somewhat dampened.; individual measurements are considerably more variable,[145] rendering fraught individual comparisons with the proposed pathological annualized cut-off value of -0.4% brain volume loss.[146]

Due to the limitations of conventional imaging techniques, there has been considerable interest in the development and application of novel acquisition and post-processing methodologies that can shed further light on the underlying pathophysiology of MS.

Neuroimaging

Diffusion weighted imaging

Diffusion-weighted imaging (DWI), long a mainstay in the clinic for assessment of stroke, has gained attention over the last few decades to investigate CNS tissue microstructure. Although the technical details are beyond the scope of this thesis, the general concept is that diffusion-sensitizing gradients are added to the pulse sequence to probe the motion of water. Due to the Brownian motion of water molecules, the MRI signal will change as a result of diffusion, which can then be used to infer properties of the local water environment. For example, water located near axonal myelin shows restricted diffusion when compared to ventricular CSF. Although one could in theory obtain information using only one diffusion encoding gradient, this is rarely done in practice. Instead, many diffusion-sensitizing gradient directions are acquired, which then allows for post-processing techniques that can better inform on the tissue microstructure.

One of the most common DWI post-processing techniques is that of diffusion tensor imaging (DTI). In this case, at least six different diffusion directions are required, but typically a larger number is acquired to improve reliability and/or facilitate better post-processing approaches. DTI processing provides a number of different types of quantitative maps, such as fractional anisotropy (FA), mean diffusivity (MD), among others. Numerous studies have used these measures

to assess tissue damage both within focal lesions[147,148] as well as within the NAWM.[147] In general, lesions are characterized by lower FA and increased MD (both indicative of increased damage) compared to the NAWM[149,150] while similar findings have been reported in the NAWM of MS patients compared to that in healthy controls. [147]Interestingly though, it is typically DTI parameters measured within the NAWM that show stronger associations with clinical outcomes[147,151] as well as with gray matter damage,[147,152,153] further highlighting the importance of non-conventional imaging approaches.

Functional MRI

Another non-conventional technique that has become increasingly popular is that of functional MRI (fMRI). Traditionally, fMRI experiments were set up such that participants would repeatedly perform a certain task (e.g., finger tapping, cognitive test) followed by a period of rest. This is then repeated a number of times, all while the MRI scanner continues to acquire a series of images sensitive to changes in T2*. Afterwards, a researcher can then perform statistical modeling to determine which areas of the brain "activate" while engaged in the task. The underlying principle is that areas of the brain that are active will end up receiving more blood. The increased cerebral blood flow and volume results in higher oxyhemoglobin, and consequently in reduced paramagnetic deoxyhemoglobin, finally leading to an increase in signal intensity . [154] As a result, this type of imaging contrast is typically referred to as blood oxygen level dependent. fMRI findings in MS are diverse and often dependent on the specific task being investigated. However, the general trend is that MS patients tend to activate in a more widespread pattern compared to healthy controls in addition to having larger activation peaks.[4,155,156] Both of these aspects can be interpreted in the context of MS patients recruiting additional brain areas to complete a certain task as well as requiring stronger "effort" in order to compensate for MS-related tissue injury.[156]

More recently, much of the research using fMRI has been dedicated to so-called resting state-fMRI (rs-fMRI), where participants do not engage in any given task but are instead just told to stay awake and think of nothing in particular. With these type of acquisition, post-processing can be

used to uncover functional networks throughout the brain that correspond to areas that spontaneously activate together.[157] This in turn allows for one to investigate functional connectivity and how it changes as a result of the disease. Resting state-fMRI is attractive since it is simpler from an acquisition point of view given that patient compliance is no longer an issue. However, these types of studies can be considerably more complex to interpret as functional connectivity changes do not appear to follow a linear trajectory as a result of the disease.[158] For example, in the initial stages of the disease, increased functional connectivity may represent an adaptive change[159] in an attempt to compensate for tissue damage. However, as the disease progresses, these changes may end up being maladaptive[160] as compensatory mechanisms are exhausted.

Iron imaging

Pathological, as well as MRI, studies have revealed that MS is also characterized by altered iron homeostasis. Changes have been noted throughout the gray matter as well as within lesions and the NAWM. As of now though, the exact cause of iron dyshomeostasis remains elusive and there remains still some debate as to whether it is a primary effect of the disease or merely an epiphenomenon secondary to tissue injury.[6] Nevertheless, iron itself plays an essential role in various processes in the brain, including neurotransmitter synthesis,[161] myelination, mitochondrial energy production,[162] DNA synthesis and repair,[163] among others. As such, it is perhaps not surprising that iron levels in the brain are affected in a disease such as MS that causes widespread damage throughout the CNS.

Much of the current knowledge about iron in the brain with respect to MS comes from neuroimaging studies. A number of techniques have been developed for quantifying iron content in vivo. Some of the early studies used a simple semi-quantitative measure of T2-weighted hypointensity of the deep gray matter,[164] showing associations with clinical outcomes.[164,165] Another technique is the so-called field-dependent R2 increase, which is rather cumbersome as it relies on acquiring images from scanners with two different field strengths.[166] More recently, most of the attention in the MS field has been focused on multi-echo gradient sequences, which allow for

the calculation of R2* maps, phase maps, as well as quantitative susceptibility mapping (QSM) maps. As of now, several studies have consistently shown increased concentrations of iron in the deep gray matter of MS patients,[1,167-169] although there is some work that suggests the opposite may be true, particularly within the thalamus.[1,170] Iron sensitive imaging has also played an important role in characterizing chronic, active lesions in the white matter. A subset of MS lesions presents with a paramagnetic rim that can be visualized using such techniques. These lesions are characterized by a lesion that is essentially silent at the core, but the periphery of which contains activated microglia and are thought to represent chronic active inflammation.[171]

Magnetization transfer imaging

Magnetization transfer imaging (MTI), in general, reflects tissue integrity. The theoretical background of this technique resides in the property of macromolecules-bound protons to absorb energy derived by radiofrequency pulses and then transfer it to nearby free-water protons that become then saturated. When another radiofrequency pulse is directed towards free-water protons already partially saturated, the derived signal will be reduced, and the difference can be quantified in terms of magnetization transfer ratio (MTR). MT-weighted images can be used to visually detect structural changes before they become apparent on conventional sequences, such as T2-weighted sequences, and predict the appearance of Gd+ lesions.[172] In this context, MTI can be used to monitor MS lesion evolution, and it is highly correlated to the sensitivity of T2-weighted lesion load and to the number of lesions detected with fluid-attenuated inversion recovery images in high-field MRI (7 Tesla), as such being a marker of neuroinflammation. [173,174]

Moreover, MTR changes are sensitive to myelin content, and reliably reflect demyelination and remyelination both in lesions and normal appearing white matter. [175]

Optical coherence tomography

Optical coherence tomography (OCT) is a relatively novel imaging technique that exploits infra-red light to analyze and quantify thickness and areas of different retinal layers.[176] It has several applications in multiple medical branches, such as cardiology, oncology, and dermatology as it is

inexpensive, non-invasive, well-tolerated and highly reproducible. It has also been applied to the study of MS, as it allows to study retinal layers in which both ganglion cells and axons reside.[177] More specifically, the most commonly used OCT measures are the peripapillary retinal nerve fiber layer thickness (pRNFLT) and the ganglion cell and inner plexiform layer thickness (GCIP), reflecting respectively the integrity of axons and neuronal cellular bodies. Thinning of these retinal layers are usually associated to retinal neuroaxonal degeneration that can happen as a consequence of acute optic neuritis (ON), but also in the context of MS, without a history of ON. Some studies have described an association between OCT markers and MRI atrophy measures,[178,179] as well as between pRNFLT and clinical measures of short-term and long-term disability and cognitive impairment.[180-183]

Optical Coherence Tomography has also been useful in monitoring other dysimmune CNS diseases, such as the neuromyelitis optica spectrum disorders (NMOSD), a pathological entity characterized by the presence of a pathogenetic biomarker, namely antiaquaporin-4 antibody, and a severe tissue damage preferentially located at the spine and optic nerve level [184,185]

Laboratory biomarkers

Besides MRI markers, there has been a long search for laboratory biomarkers able to facilitate the diagnosis, or helpful in early prediction of long-term evolution. Whereas in other dysimmune CNS diseases, such as NMOSD[185] and myelin oligodendrocyte glycoprotein antibody associated disorders (MOGAD),[186] biomarkers were identified and in the former case helped clarifying the pathogenesis of the disease, to date, there is no specific biomarker for MS. For a long time, the only biomarker considered in the MS field was the presence of oligoclonal bands (OB) in the CSF, but they have a low specificity as they simply indicate the presence of a dysimmune inflammatory reaction within the CNS. They are very sensitive though, as up to 95% patients with MS are OB+.[187]

However, despite intensive research, the antigen towards which the Immunoglobulins G that are visualized as OB remains unidentified. Their widespread presence in the CSF of MS patients led to their inclusion in the diagnostic criteria by Poser et al.[12] Furthermore, recent studies showed their predictive role for conversion from RIS to clinically definite MS, as RIS patients positive for OB converted faster to CIS and MS than patients OB-. [188]

Neurofilaments (Nfl) are a major neuronal component, released upon neuroaxonal damage. They are detected in up to 78% of MS patients, and are significantly associated with the disability level and the presence of a recent disease exacerbation,[189] as well as with the presence of a progressive form,[190] and global and regional brain atrophy [191,192] as early as in CIS.[192]

Some other proteins have been analyzed in the attempt of identifying lab biomarkers of disease progression, such as the myelin basic protein, a marker of WM demyelination, neuron specific enolase (NSE), a marker of neuronal damage, but none of them has shown enough specificity.[193] Moreover, acquiring CSF markers is not ideal, as the invasiveness of the maneuver (lumbar puncture) needed to obtain CSF for analysis prevents their use for routine monitoring purposes. Recently, the comparative dosage of serum and CSF neurofilament light (NfL) chain, revealed that serum Nfl (sNfL), although with a 40-fold decrease with respect to CSF amount, is still a reliable marker for WM injury,[194] with a strong correlation between CSF and serum levels [192,195]

Further studies have reported significantly higher sNfL levels in MS patients than in healthy controls as well as an association between sNfL and EDSS changes, Gd-enhancing lesions and neuropsychological outcomes, [192,196] making them a very promising, easily obtainable biomarker for disease activity and progression. When prospectively assessed, sNfL levels were strongly associated with both new/enlarging [197,198] and Gd+ lesions,[198] as well as with global and regional brain atrophy and spinal cord volume loss over 5 years, [199,200] and even 10 years.[201]

Moreover, patients with MS under disease modifying treatment had significantly lower levels of sNfL,[192,195] and switching from a “first-line” therapy to a more efficacious one was associated with a significant decrease of sNfL.[202] A recent study repeatedly quantified sNfL in a large population of

patients starting new disease modifying treatments (DMT) and detected an overall decrease of sNfL after treatment initiation, but with relevant differences among therapies. [203] SNfL could therefore be used also to monitor treatment response in the context of RCTs and, possibly, in the routine clinical practice [204]

The current knowledge on the pathogenetic mechanisms driving MS has drastically increased, in large part due to the advent of advanced MRI techniques that allowed to acquire relevant in vivo information from the earliest disease stages. Yet, several issues need to be clarified, among which are the relationship between neuroinflammation and neurodegeneration and a better characterization of disease progression. With this background, the overarching aim of the manuscripts here enclosed was to apply multimodal approaches in conjunction with sophisticated imaging techniques to identify markers of disease progression and study the potential role of neuromotor rehabilitation as a non-pharmacological treatment capable of counteracting the progression of MS.

Brief overview of disease modifying treatments in MS

As mentioned earlier, the first pharmacological treatment officially approved for MS was Interferon-ß (IFN) 1b, for its proven efficacy in reducing the relapse rate.[20] Both Interferon-ß 1b and 1a are currently available as injectable therapies with an administration frequency that ranges from 3/week to bimonthly, depending on the specific drug. Their mechanism of action is complex, including modulation of the production of pro- and anti-inflammatory cytokines.[205] They are characterized by a high safety profile, with only local reactions and flu-like syndrome as the most common side effects, to the point that they are approved also for use in pregnant women. The other injectable therapy, glatiramer acetate (GA), is currently available in two different dosages and frequency of administration (daily or 3/week), although a depot formulation with a less frequent need of injection is under investigation. GA is a synthetic analogue of the myelin basic protein, a major constituent of myelin sheaths, that acts by modulating the cytokine pattern and competing

with different antigens to their presentation to T-lymphocytes. Furthermore, it stimulates the production of brain-derived nerve growth factor, potentially promoting neuro-regeneration. The only side effects are represented by cutaneous reactions in the injections site.

More recently some oral therapies have also become available for the treatment of MS, overcoming the psychological and physical issues related to the need for injections. Dimethyl fumarate (DMF) has been approved for RRMS based on its efficacy in reducing Gd-enhancing lesions, new/enlarging T2 lesions and annualized relapse rate.[206] Again, DMF effectiveness resides in the capability to modulate the immune system reducing the production of proinflammatory cytokines, inflammatory mediators in activated microglial cells,[207] downregulating oxidative stress. Most common side effects are represented by gastrointestinal symptoms and flushing. Fingolimod (FTY) and the more recent compounds Ozanimod and Siponimod are antagonists of the sphingosine-1 phosphate receptor, which is commonly found in several cell types, among which T-lymphocytes. They share a similar mechanism of action, which consists of preventing egress of T-lymphocytes from lymphoid tissue, thus reducing the amount of autoaggressive T-lymphocytes circulating in bloodstream and entering the CNS.[208] FTY showed higher efficacy than IFNß-1a in reducing relapse rate (52% vs 32%), and appearance of new Gd-enhancing lesions.[209] The most common side effects are mild tiredness, diarrhea, stomach ache and altered liver functions, but some cases of varicella-zoster virus reactivation have been reported,[210] as well as few cases of progressive multifocal leukoencephalopathy (PLM).[211] The first dose administration requires EKG monitoring for at least 6 hours for some reported cases of bradyarrhythmia, and rare cases of sudden death.

Teriflunomide is another oral agent approved in MS, whose mechanism of action is not completely understood but seems to be based on the inhibition of pyrimidines in rapidly proliferating cells such as T- and B lymphocytes thereby reducing their reactivity to autoantigens.[212] Compared to placebo, teriflunomide reduced annualized relapse rate, disability progression and several MRI markers of neuroinflammation.[213] Teriflunomide is a generally well tolerated drug, with very few, reversible side effects (e.g. diarrhea, nausea, hair loss, altered liver enzymes).

Cladribine is another oral therapy, but it is characterized by a peculiar administration: it only needs to be taken twice, with the two short cycles 1 year apart from each other. Cladribine acts by depleting both T-and B-lymphocytes and is recommended as a first-line therapy for highly active MS forms. Cladribine showed efficacy in reducing the annualized relapse rate (ARR) by 57.6% vs placebo, the number of Gd+-enhancing lesions as well as new T2 lesions.[214] Side effects include upper respiratory or genitourinary infections, lymphopenia and some other minor symptoms.
Natalizumab (NTZ) is the first monoclonal antibody approved for use in treating MS. It acts by binding to α4β1-integrin receptors thereby inhibiting leukocyte migration into brain tissue.[215]It is characterized by a rapidly acting anti-inflammatory activity, and a very high efficacy in reducing relapse rate, Gd+ enhancing lesions, as well as T2 lesions (9.6 lesions/patient in the placebo group vs 0.7 in the NTZ group 6 months after treatment initiation).[216] The most severe complication of NTZ treatment corresponds to the potential development of Progressive multifocal leukoencephalopathy (PML), an opportunistic infection leading to encephalitis. It is caused by the ubiquitous John Cunningham virus normally quiescent, but potentially reactivated by lack of immunosurveillance. The likelihood of presenting with PML after two years of treatment increases significantly and limits the possibility to use NTZ for undetermined periods of time.[217]
A better understanding of the MS-related pathogenetic mechanisms has highlighted the role of B lymphocytes, both in the periphery and within the CNS, sustaining the chronic compartmentalized inflammation within the CNS. Ocrelizumab is among the newest pharmacological compounds approved for the treatment of MS, and one of the two drugs registered for progressive MS forms.[218,219] Ocrelizumab is a humanized antiCD20 antibody that owes its efficacy to a mechanism of depletion of B lymphocytes, indirectly confirming the crucial role of these latter in disease evolution. Rituximab is another antiCD20 antibody that acts similarly, but it is not approved for MS yet, thus being administered only in selected cases and in an off-label regimen. [220]
Alemtuzumab is an anti-CD52 antibody that acts by depleting both T- and B-lymphocytes as well as monocytes. It is a very effective treatment that, for the wide immune depleting mechanism of

action, is usually reserved as a second- or third-line therap.[221] Post marketing studies showed the efficacy of alemtuzumab on markers of neuroinflammation as well as neurodegeneration, such as a reduction of annualized relapse rate, an EDSS decrease in 20.6% of treated patients together with a progression-free survival in 89.2% of treated patients after 2 years.[222]

The current pandemic of SARS-Cov2 has raised many questions with respect to safety of different MS drug treatments that were not previously considered, mainly with respect to ability of the immune system to build an effective response to an infectious agent never encountered before, and to the possible interactions between MS treatments and vaccines. A recent study, however, has shown that drugs are in generally safe and, but for antiCD20 therapies, do not impact on the overall risk of hospitalization, intensive care unit admissions and death.[223] However, among all the available treatments, ocrelizumab and rituximab were associated with the greater risk of hospitalization and Intensive Care Unit admission, and rituximab was associated with a higher risk of artificial ventilation.[224]

In contrast, MS patients under different types of treatments were studied, to quantify their humoral and cellular response after anti-COVID vaccination: as expected, patients treated with ocrelizumab had a lower humoral response, whereas patients treated with FTY had the lowest frequency of T-cell response. Therefore, patients affected with MS show undoubtedly the presence of at least a partial response of the immune system, even in patients treated with disease modifying treatments, advocating for the more systematic use of vaccines.[225]

Non-pharmacological treatments: neuromotor rehabilitation and neuroplasticity

The last Global Burden of Disease Study showed that neurological diseases are the primary cause of disability-adjusted life years (DALYs), and whereas MS-related mortality has significantly decreased in the last decade, age-related DALYs have not changed.[35] Moreover, identified genetics and environmental risk factors explain less than 10% of DALYs burden in most neurological

conditions, including MS.[34,35] Therefore, on the one hand primary prevention is not feasible, and on the other currently available pharmacological therapies have limited efficacy in slowing down or reverting disease progression. Thus, strategies capable of mitigating progressive disability gain are urgently needed.

Neuroplasticity is the ability of the brain to modify itself at a structural and functional level in response to aging, learning and environmental stimuli.[226-231] Experimental and clinical studies have demonstrated the usefulness of physical activity in MS, both at an osteoarticular/muscular and cardiac level, but also promoting positive neuroplasticity.[232-240] The United States National MS Society has endorsed the positive role of physical exercise, or an active lifestyle, recommending at least 150 minutes/week to patients affected by MS.[241]

Both experimental studies on animal models of MS and the use of advanced MRI techniques applied to patients undergoing neuromotor rehabilitation have shed some light on the mechanisms eliciting neuroplasticity.[239,242] In particular, rehabilitation stimulates remyelination and neuroaxonal regeneration, as demonstrated in animal studies, while it reduces tissue damage, favoring the recovery of tissue integrity in patients with MS.[243,244].

Several rehabilitative studies concordantly reported a beneficial effect on both objective and subjective clinical outcomes, such as fatigue, balance, endurance, strength and in general quality of life.[245-249] A recent systematic review described the effect of neuromotor rehabilitation on brain neuroplasticity, reporting that motor rehabilitation has the potential to favorably impact brain neuroplasticity.[155] However many factors, such as individual disease stage and duration, as well as rehabilitation type and duration, influence the type and degree of cerebral response. Moreover, the few studies investigating the relationship between neuroplasticity and MS by means of advanced MRI techniques have been limited by different methodological pitfalls regarding the study design, the control group, and more generally by the heterogeneity of rehabilitative methods and MRI markers applied.[155]

The importance of better understanding how to promote neuroplasticity through neurorehabilitation, thus counteracting the effect of a chronic tissue damage, resides also in the concept of "Medxercise" (Medicine+exercise). Experimental studies have shown that the simultaneous administration of medications promoting remyelination and physical exercise favoring the release of growth factors, neuro- and synaptogenesis, has an additive positive effect on disease progression.[250]

Despite the clinical evidence of a positive effect of physical exercise on both motor, psychological and cognitive outcomes, the lack of robust markers to quantify the benefit and investigate the underlying neural mechanisms makes it necessary to plan further larger and well-designed studies that would allow to tailor rehabilitative strategies on patients' needs and abilities, with a final relevant effect on disease evolution.

Gaps in knowledge

As described in this Introduction, the last decades have been characterized by a tremendous advancement in the knowledge of the disease, its pathogenetic mechanisms, related risk factors and prognostic factors. Nonetheless, some crucial questions are yet to be answered, among and above others the ones pertaining to the relationship between neuroinflammation and neurodegeneration and how their interaction leads to irreversible disability accrual. To clarify these aspects is of paramount importance as it would lead to the identification of novel, more targeted therapies with effective neuroprotective properties. The current project helps unraveling the mystery, by applying different conventional and advanced MRI techniques enabling to capture multiple structural, functional and metabolic processes undergoing MS course, allowing for a more global, complementary and thorough view of the MS-related pathophysiology.

Chapter 2. Hypotheses

MS is a complex disease characterized by the presence of numerous factors contributing to disease progression, including, but not limited to, lesion formation, chronic tissue degeneration, and iron dyshomeostasis.

Applying multimodal imaging approaches in two of the different disease phenotypes (relapsing-remitting and progressive MS) will allow us to capture different aspects of disease progression, potentially assessing the underlying differences in pathophysiology. This in turn will help defining the possible association between several MRI markers and clinical disability measures, ultimately facilitating the understanding of processes that can be the target of new therapeutic approaches as well as of neurorehabilitation. Neurorehabilitation is a non-pharmacological tool thought to be able to facilitate brain reorganization through neuroplastic mechanisms.

Conventional MRI techniques are not sensitive enough to capture disease progression, therefore we need to apply other advanced imaging approaches to evaluate disease evolution over time. More specifically, the combination of imaging techniques able to investigate structural and functional features of the cerebral tissue affected by MS as well as specific metabolic aspects would allow a better characterization of different disease phenotypes and help understanding the contribution of neuroinflammation and neurodegeneration to disease progression. The overall hypothesis driving the research projects here included regards the possibility to identify MRI markers predictive of disease progression, which in turn leads to the transition from relapsing remitting forms of disease to secondary progressive forms of MS. The specific hypotheses underlying the different studies here reported are as follows:

1) Histopathological data first and then MRI data have consistently shown the presence of altered iron content in different CNS compartments, in MS as well as in other neurodegenerative diseases. Whether this is the cause or a consequence of tissue damage has yet to be defined. Recent evidence of the key role of grey matter in MS progression has led to investigate the presence of tissue damage in deep grey matter structures, such as basal ganglia and the thalamus. The latter is of particular interest, as it is one of the main brain hubs, connecting cortical and subcortical structures. Thalamic iron content has been previously investigated ion MS with discrepant results likely due to the application of different MRI techniques. Considering it an intriguing subject with potential relevant

consequences, we decided to use quantitative susceptibility mapping to analyze deep grey matter iron content. Both a region-of-interest based and a voxelwise-based approach were applied, associating quantitative measures of susceptibility to clinical measures of disability in different MS phenotypes. The working hypothesis of the study was to verify whether iron content was altered in different DGM structures and how iron dyshomeostasis was associated to clinical disability and progressive forms of MS, potentially making iron-related markers suitable for monitoring disease evolution over time.

2) The previous identification of a novel MRI marker, so-called atrophied lesion volume, derived from the application of conventional MRI techniques and related to disease evolution, is of notable scientific interest. The histopathological correlate of atrophied lesion volume is not clear but it seems to be the result of irreversible tissue loss. Based on these previous findings and bearing in mind the need to identify reliable and reproducible measures of disease progression, we performed a study applying atrophied T2-lesion volume together with other MRI markers, biomarkers and cognitive measures reflective of disease progression. We hypothesized that atrophied lesion volume could correlate with multimodal measures of neurodegeneration, confirming its potential role as a marker of neurodegeneration.

3) Optical coherence tomography (OCT) is emerging as an alternative imaging technique for the study of neuroaxonal damage, with several advantages with respect to advanced MRI techniques in terms of costs, accessibility, and ease-of-use. Several studies have investigated the correlation between OCT markers and brain volumetric measures, confirming the association between the entity of retinal and brain neuroaxonal damage. With this background, we planned to study the association between OCT and a serum biomarker of neuroaxonal damage in different MS phenotypes, to further verify the role of OCT measures as markers of neurodegeneration.

4) Neuromotor rehabilitation is currently implemented in clinical practice in several neurological diseases, including MS, with different approaches aimed at recover lost functions and slow down disability accrual. The neurobiological explanation of the efficacy of neuromotor rehabilitation resides in its ability to promote brain neuroplasticity, but the exact mechanisms through which rehabilitation exerts its effects are still unclear. We planned a study combining different advanced MRI techniques to evaluate the effects of intensive neuromotor rehabilitation on structural and functional connectivity.

Chapter 3. Brain Iron at Quantitative MRI is associated with disability in multiple sclerosis

Abstract

Purpose To study deep gray matter susceptibility in multiple sclerosis (MS) by using quantitative susceptibility mapping (QSM) and to assess the relationship between susceptibility and clinical disability. **Materials and Methods** For this prospective study between March 2009 and November 2013, 600 participants with MS (452 with relapsing-remitting MS and 148 with secondary progressive MS) and 250 age- and sex-matched healthy control participants were imaged with 3.0-T MRI to measure magnetic susceptibility. Deep gray matter susceptibility (in parts per billion) was analyzed by using region of interest and voxelwise methods. QSM and MRI volumetric differences between study groups and associations with clinical outcomes were assessed. Analysis of covariance, multivariable linear regression, and voxelwise analyses, controlling for age and sex, were used to compare study groups and to explore associations between MRI and clinical outcomes. **Results** Compared with control participants, participants with MS presented with lower thalamic susceptibility (-7.5 ppb vs -1.1 ppb; $P < .001$) and higher susceptibility of basal ganglia (62 ppb vs 54.8 ppb; $P < .001$). Lower thalamic susceptibility was associated with longer disease duration ($\beta = -0.42$; $P = .002$), higher degree of disability ($\beta = -0.64$; $P = .03$), and secondary-progressive course ($\beta = -4.3$; $P = .009$). Higher susceptibility of the globus pallidus was associated with higher disability ($\beta = 2$; $P = .03$). After correcting for each individual structural volume in voxelwise analysis, lower thalamic susceptibility and higher susceptibility of the globus pallidus remained associated with clinical disability ($P < .05$). **Conclusion** Quantitative susceptibility mapping (QSM) suggests that altered deep gray matter iron is associated with the evolution of multiple sclerosis (MS) and on disability accrual, independent of tissue atrophy

Chapter 4. A multimodal approach to assess the validity of atrophied T2-lesion volume as an MRI marker of disease progression in multiple sclerosis

Abstract

Background: Atrophied T2-lesion volume (LV) is a novel MRI marker representing brain-lesion loss due to atrophy, able to predict long-term disability progression and conversion to secondary-progressive multiple sclerosis (MS).

Objective: To better characterize atrophied T2-LV via comparison with other multidisciplinary markers of MS progression

Methods: We studied 127 MS patients (85 relapsing-remitting, RRMS and 42 progressive, PMS) and 20 clinically isolated syndrome (CIS) utilizing MRI, optical coherence tomography, and serum neurofilament light chain (sNfL) at baseline and at 5-year follow-up. Symbol Digit Modalities Test (SDMT) was obtained at follow-up. Atrophied T2-LV was calculated by combining baseline lesion masks with follow-up CSF partial-volume maps. Measures were compared between MS patients who developed or not disease progression (DP). Partial correlations between atrophied T2-LV and other biomarkers were performed, and corrected for multiple comparisons.

Results: Atrophied T2-LV was the only biomarker that significantly differentiated DP from non-DP patients over the follow-up ($p = 0.007$). In both DP and non-DP groups, atrophied T2-LV was associated with baseline T2-LV and T1-LV (both $p = 0.003$), absolute change of T1-LV (DP $p = 0.038$; non-DP $p = 0.003$) and percentage of brain volume change (both $p = 0.003$). Furthermore, in the DP group, atrophied T2-LV was related to baseline brain parenchymal ($p = 0.017$) and thalamic ($p = 0.003$) volumes, thalamic volume change and follow-up SDMT (both $p = 0.003$). In non-DP patients, atrophied T2-LV was significantly related to baseline sNfL ($p = 0.008$), contrast-enhancing LV ($p = 0.02$) and percentage ventricular volume change ($p = 0.003$).

Conclusion: Atrophied T2-LV is associated with disability accrual in MS, and to several multimodal markers of disease evolution.

Chapter 5. Serum neurofilament light chain and optical coherence tomography measures in MS: A longitudinal study

Abstract

Objective: To study the association between serum neurofilament light chain (sNfL) and multiple optical coherence tomography (OCT) measures in patients with MS and healthy controls (HCs).

Methods: In this prospective study, 110 patients with MS were recruited, together with 52 age- and sex-matched HCs. Clinical evaluation and spectral domain OCT and sNfL were obtained at baseline and after 5.5 years of follow-up. Nested linear mixed models were used to assess differences between MS vs HC and associations between sNfL and OCT measures. Partial correlation coefficients are reported, and *p* values were adjusted for the false discovery rate.

Results: At baseline, peripapillary retinal nerve fiber layer thickness (pRNFLT) and macular ganglion cell and inner plexiform layer thickness (mGCIP) were significantly lower in MS than HC both in MS-associated optic neuritis (MSON) ($p = 0.007$, $p = 0.001$) and nonaffected MSON (n-MSON) eyes ($p= 0.003$, $p = 0.018$), along with total macular volume (TMV) in n-MSON eyes ($p = 0.011$). At follow-up, MS showed significantly lower pRNFLT, mGCIP, and TMV both in MSON

Conclusions: This study confirms the ability of sNfL to detect neurodegeneration in MS and advocates for the inclusion of sNfL and OCT measures in clinical trials.

Classification of evidence: This study provides Class III evidence that sNfL levels were associated with MS neurodegeneration measured by OCT.

Chapter 6. Effects of motor rehabilitation on mobility and brain plasticity in multiple sclerosis: a structural and functional MRI study

Abstract

Background: Rehabilitation seems to promote brain plasticity, but objective measures of efficacy are lacking and there is a limited understanding of the mechanisms underlying functional recovery.

Objective: To study functional and structural brain changes induced by gait rehabilitation.

Methods: We enrolled MS inpatients (EDSS 4.5-6.5) undergoing a 4-week neurorehabilitation. Several clinical measures were obtained, including: 2-min walk test (2MWT), dynamic gait index (DGI), Berg balance scale (BBS). Furthermore, motor-task functional MRI (fMRI) of plantar dorsiflexion, resting state fMRI, and regional diffusion tensor imaging (DTI) metrics were obtained. All the assessments were performed at baseline (T0), after the end of the rehabilitation period (T1) and 3 months later (T2).

Results: Twenty-nine patients were enrolled at T0, 26 at T1, and 16 completed all timepoints. At T1, there was a significant improvement of 2MWT, DGI, and BBS scores, along with a reduced extent of the widespread activation related to the motor task at the fMRI and an increased functional connectivity in the precentral and post-central gyrus, bilaterally. None of these changes were maintained at T2.

Conclusions: Our findings show a short-term beneficial effect of motor rehabilitation on gait performances in MS, accompanied by brain functional reorganization in the sensory-motor network.

Chapter 7. Discussion

The studies presented here had the overall common aim to identify and describe pathogenetic features of different disease phenotypes and in particular to characterize progressive MS forms, therefore contributing to identify markers of disease progression.

Even though each study here included presents results derived from the application of different imaging modalities, they are at the same time complementary to each other, as they all provided information useful to characterize different phases of MS evolution, and to identify features typical for progressive forms of MS.

Brain Iron at Quantitative MRI is associated with disability in multiple sclerosis

The specific hypothesis of the first paper was that global and regional susceptibility, as a marker of iron dyshomeostasis, is more severely altered in the deep grey matter structures in progressive forms of MS.[1] A growing body of evidence points at the deep grey matter (DGM) structures as an anatomical region with a crucial role in determining physical and cognitive impairment in MS and suggests that iron dyshomeostasis might have a major role in determining DGM tissue damage. Therefore, we explored magnetic susceptibility of DGM structures by applying quantitative susceptibility mapping (QSM) of the thalamus and basal ganglia, and associated DGM magnetic susceptibility to clinic-demographic measures such as disease duration and EDSS in different disease phenotypes. Moreover, we dedicated a review reported in the Appendix to discuss the role of iron in MS and other neurological diseases, with a particular focus on iron dyshomeostasis in DGM structures. [6]

Clinical and theoretical implications

Our results showed that both thalamic and basal ganglia susceptibility, besides being significantly altered in the MS group as a whole, show statistically significant differences in SPMS

vs RRMS. The region-of-interest based analysis demonstrated that thalamic susceptibility is lower and GP susceptibility tends to be higher in SPMS. Moreover, SPMS showed a significantly lower thalamic susceptibility together with a significantly higher susceptibility of the caudate, putamen and GP with respect to RRMS in the voxel-wise analysis. Lower thalamic susceptibility was associated with higher EDSS scores even after correcting for individual structural volumes. Both the ROI-based analysis and the voxelwise analysis confirmed a more pronounced iron dyshomeostasis in progressive forms of diseases, and a significant association between altered iron content within the thalamus and the globus pallidus and disease duration and clinical disability.

Overall, the study confirmed a pathogenetic role of iron dyshomeostasis, which contributes to accrual of disability at least partially independently from deep GM tissue loss.

Shortcomings

The cross-sectional nature of the study, together with relatively long disease duration of enrolled MS patients, did not allow definite conclusions on the temporal evolution of iron abnormalities, nor to establish a causal relationship between altered iron content and tissue loss. However, the interaction effect between disease course and thalamic susceptibility, together with the more pronounced iron alterations in SPMS, allowed us to hypothesize a dynamic iron impairment evolving over time with different features in each MS phenotype. The other limitation of the study is related to the use of FIRST to calculate regional gray matter volumes, so that partial volume correction was not included into the resulting structural volumes. As such, higher partial volume averaging with progressive atrophy may have impacted on our results

A multimodal approach to assess the validity of atrophied T2-lesion volume as an MRI marker of disease progression in multiple sclerosis

Conventional MRI sequences are routinely used in clinical practice and as the gold standard for capturing radiological activity in randomized clinical trials. Although traditional measures derived by conventional MRI sequences, such as lesion loads, have a limited ability to predict clinical disability, they are easily obtainable and standardized, making results at least somewhat comparable

between different studies. Therefore, in the second paper, we used conventional T2-weighted sequences in a longitudinal fashion to build a novel biomarker, so-called atrophied T2-lesion volume, which reflects the destruction of lesional tissue in the brain of MS patients and we analyzed the association between the former and other conventional measures of lesion load as well as atrophy measures. [2]

Clinical and theoretical implications

The findings of this study confirmed and expanded previous results. It showed that atrophied T2-LV was significantly higher in MS patients progressing over time with respect to stable MS patients, and was the only MRI marker able to differentiate disease-progressed (DP) versus non-DP patients. Furthermore, atrophied T2-LV was significantly associated with T1- and T2-weighted lesion loads, absolute changes in T1-volumes and global brain volume measures both in SPMS and in DP patients. These findings confirmed the potentially relevant role of this MRI marker in capturing some pathogenetic aspects of disease progression. Interestingly, atrophied T2-LV was also associated with thalamic volume and thalamic volume changes in DP patients, supporting the relevant role that thalamic dysfunction might have in the pathogenesis of MS. Notably, atrophied T2LV was also related to a cognitive marker measured at follow-up and to a widely used biomarker, namely sNFL. Even though sNFL reflects both neuroaxonal damage and neuroinflammation, as its levels are higher both preceding and during acute relapses and along the course of the disease, it is considered an easily accessible marker, sensitive to cerebral tissue damage. Cognitive dysfunctions in MS are known to occur since the early disease phases and become clinically relevant over time, with a significant impact on the social and professional life of MS patients. The association of these markers with atrophied T2LV, more pronounced in patients showing a progression of clinical disability, further confirms the utility of atrophied T2LV as a marker of neurodegeneration.

Shortcomings

The major limitation of this novel marker is related to the technical challenges in properly detecting cortical lesions, so that atrophied T2-LV mostly reflects the periventricular lesional component and not the global lesional burden.

Another limitation if this study, shared by other studies here included, is related to the small sample size of progressive forms of MS, that might have hampered not been assessed at baseline, rendering it difficult to define a possible association between atrophied T2LV and the baseline cognitive status

Serum neurofilament light chain and optical coherence tomography measures in MS: A longitudinal study

The third study applied OCT as an alternative imaging technique, hypothesizing that OCT markers such as Retinal Nerve Fiber Layer Thickness and Ganglion Cell/Inner Plexiform, layer thickness putative biomarkers of neuronal loss, are lower in progressive MS forms and associated with laboratory biomarkers of neurodegeneration.[3]

Clinical and theoretical implications

The analysis was carried out at two levels: first, comparing MS patients with healthy controls, and then, within the MS group, at an "eye level", comparing eyes affected by optic neuritis (ON eyes) with eyes that were never affected by optic neuritis (n-ON). The MS group as a whole showed a significant reduction of several OCT markers with respect to healthy controls, both in ON-affected and non-affected eyes. These changes were detected both at baseline and follow-up, together with significant associations between sNfL and pRNFLT and mGCIP in n-ON eyes. However, the results in subgroups of different MS phenotypes were less striking. In particular, in the group of eyes not affected by ON, baseline mGCIP showed a tendency to be lower in PMS whereas in the ON group, baseline INL was significantly higher in PMS versus RRMS. With respect to follow-up OCT measures, in the n-ON group, pRNFLT tended to be lower and INL tended to be higher in PMS, without reaching statistical significance in any of the comparisons. There were no significant correlations between sNfL, a marker of neurodegeneration and OCT

measures in the PMS group, but sNfL was significantly associated with both pRNFLT and mGCIP in the whole MS cohort and in RRMS. The small sample size might explain the lack of significant results in the subgroups, but some interesting conclusions can still be drawn, as both sNFL, pRNFLT and mGCIP were significantly altered as well as significantly associated in MS as compared to healthy controls,thus confirming their potential role as markers of neurodegeneration.

Shortcomings

The small sample size, in particular with respect to MS-ON eyes, might have had a relevant negative role, affecting the differences between populations and all the partial correlations between sNfL and OCT measures. Larger studies are needed to build on the current findings. Furthermore, visual acuity and color vision information are missing, and even though the association between visual function and OCT measures was not one of the aims of the study, this information might have given a more complete clinical picture of the subjects enrolled in project.Effects of motor rehabilitation on mobility and brain plasticity in multiple sclerosis: a structural and functional MRI study

To analyze the effect of neurorehabilitation on neuroplasticity, as a non-pharmacological treatment potentially able to counteract progressive tissue damage, in the last study presented in this thesis we applied diffusion tensor imaging (DTI) to analyze microstructural tissue properties; functional MRI (fMRI) to analyze activity pattern in response to a motor task and resting state fMRI to analyze functional connectivity within regions of interest thought to be involved in disease progression in MS. [4]

Clinical and theoretical implications

Subjective and objective clinical measures representative for balance, resistance, fatigue, significantly improved together with functional connectivity. Interestingly, the fMRI at the baseline showed a widespread bilateral activation of both infra- and supratentorial regions, confirming previous literature data showing a diffuse cortical activation in response to simple tasks as a positive adaptive plastic mechanism occurring in chronic neurological diseases. After the cycle of intensive neuromotor rehabilitation there was a global reduction of the extension of cortical activation during motor task fMRI, paralleled with an increased connectivity both in the primary motor and somatosensory cortex at the resting state fMRI. These findings, taken together, suggest

that neuromotor rehabilitation favors neuroplasticity, promoting the functional recovery of specialized areas. However, measures of structural connectivity did not show any significant change. Moreover, all the positive clinical and functional MRI changes detected after one month were lost at the following timepoint, three months after ceasing exercising. These results, while underlying the importance of neuromotor rehabilitation as a non-pharmacological treatment able to partially counteract the chronic tissue damage, confirm on the other hand the essential role of physical activity as a regular lifestyle habit, probably difficult to maintain in most MS patients moderately disabled, for several factors involving their personal, familial and socio-economic status.

Shortcomings

This study presented with three main limitations. First, the study was initially designed to compare two different rehabilitation treatments but due to financial limitations, the original sample size was significantly reduced. This issue, together with the high drop-out rate in particular with respect ot the third timepoint has likely hampered the results. The second limitation concerns the motor task, as we found it very difficult to isolate the foot dorsiflexion movement, especially considering that patients included in the study had a mild-to-moderate disability, often presenting with motor deficits at the lower limb. Thirdly, we did not recruit a control group of healthy subjects nor of MS patients not undergoing rehabilitation

To summarize, the findings here reported add a wealth of knowledge to the existing MS literature, characterizing different aspects of disease evolution over time. We showed that disease progression is related to iron dyshomeostasis in deep grey matter structures, with different mechanisms in the thalamus and within the basal ganglia. We confirmed the usefulness of a novel MRI marker such as atrophied T2-LV, which represents both lesion accrual and simultaneously occurring irreversible tissue loss, possibly associated with some other pathogenetic mechanism yet to be identified, and confirmed the role of sNfL and OCT measures as markers reflective of neuroaxonal degeneration. Finally, we analyzed the effect of motor rehabilitation on clinical and

MRI markers, supporting its usefulness as a non-pharmacological treatment able to impact on disability, even though future efforts are needed to identify how to maintain the positive effects of neuromotor rehabilitation over time.

In the last twenty years the identification of novel biomarkers, the advent of advanced MRI techniques and the availability of data derived from large longitudinal studies have dramatically increased our knowledge on MS, which in turn has led to the identification of new pharmacological treatments characterized by high efficacy on neuroinflammation. [251] However, published data concordantly report that long-term disability is mainly associated with the neurodegenerative component of the disease. Therefore, strongly needed is a better understanding of: the mechanisms underpinning neurodegeneration; and, the relationship between neurodegeneration and neuroinflammation. The original studies presented here shared the common aim of attempting to characterize different aspects of disease progression, by applying a multimodal imaging approach. In this context, iron dyshomeostasis has gained considerable interest, mainly due to the availability of advanced techniques capable of quantifying iron concentration *in vivo* throughout the brain.[6] Moreover, chronic active lesions, also known as smoldering lesions, have become a subject of numerous studies in the last 3-5 years as they have been identified as a hallmark of disease progression, and they are often characterized by a rim of iron-laden microglia. [114,116,252] Even though it is difficult to discern whether altered iron concentration is a mere epiphenomenon or has a causal role in the pathogenesis of MS, our study provided new knowledge of the potential dynamics of iron metabolism within the brain, and especially within deep GM structures. We focused on the thalamus, which is one of the main brain connectivity hubs, as several studies showed an association between altered thalamic tissue integrity and cognitive or clinical impairment. [253-255] Our results showed reduced susceptibility within the thalamus, as well as increased susceptibility within the basal ganglia, using both region of interest-based QSM and a whole brain voxel-wise approach. This was evident both in RRMS and SPMS, with the latter group presenting more pronounced iron alterations. Moreover, reduced thalamic susceptibility was associated with higher

disability and longer disease duration, supporting the clinical relevance of thalamic iron dyshomeostasis in MS, demonstrated also in histopathological studies.[256] As highlighted in a subsequent study, there is a crucial difference between iron concentration and iron content, where changes of the former might reflect reduced volumes of the areas of interest but not actual changes in the real content of iron, whereas only changes of iron content, regardless of the volume of the structure containing iron, are indicative of an active process of iron depletion or deposition. [170] In our study we overcame this potential confounding factor by correcting for structural volumes in the voxel-wise analysis, still showing a significant reduction of thalamic iron content. A possible biological explanation of this phenomenon might reside in the progressive depletion of iron-rich oligodendrocytes, that initially release iron in the extracellular space and subsequently are scavenged by activated microglia, with a consequent production of reactive oxygen species that, in turn, create an inflammatory milieu, contributing to the self-maintenance of this treacherous mechanism. Our results, together with the majority of published data on the topic, suggest that thalamic structural and metabolic impairment, with altered iron susceptibility as well as volume changes occur and develop more rapidly in the early disease stages, being sustained initially by inflammation and later by other pathogenetic mechanisms occurring both within the thalamus and as an indirect consequence of upstream and downstream pathology. [1,2,255]

Our findings, subsequently confirmed and expanded by other studies, [257-260], have led to the identification of iron-related MRI measures as potential MRI biomarkers to be included in future longitudinal studies and clinical trials testing the efficacy of therapies in progressive MS forms. [261-263] Moreover, our findings together with others were reported in a recent consensus statement published by the North American Imaging in Multiple Sclerosis Cooperative on the relevance of deep grey matter pathology in disease progression, confirming the need to include DGM MRI markers as endpoint in clinical trials as well as in clinical practice, even though some technical challenges, such as the lack of common analysis platforms limit their application nowadays.[255]

Together with a better understanding of pathogenetic mechanisms underlying disease progression, another goal of paramount importance is the identification of reliable MRI markers able to reflect different phases of MS evolution, to be applied in the scientific research field and, possibly, in clinical practice. Traditionally, lesion loads quantified both with T1-weighted and T2-weighted sequences attempted to provide useful information in this direction but the degree of correlation with clinical markers of disability was modest, as reflected by the notion of "clinico-radiological paradox". [112] There have been several attempts to explain and ameliorate the situation, mainly by including in the correlative analysis with clinical disability not only the amount of lesions, but also their location within the brain and along the corticospinal tract.[264,265] However, these processes are time consuming and often require post-processing expertise, which is not always available, especially in clinical settings. The identification of a new marker, namely atrophied T2-lesion volume, that has been the subject of one of the studies presented here, might partially overcome the aforementioned limits as it reflects neurodegeneration and derives by conventional MRI techniques currently used in clinical practice.

The transition phase from RRM to SPMS has continued to gain increased interest over the last several years, in part thanks to the studies here reported. Although currently available treatments, more sensitive diagnostic criteria, and a wider accessibility to healthcare have positively impacted on the prognosis of MS, which appears to be characterized by a milder disease course, [266] the majority of patients still convert to SPMS within 20-30 years from the diagnosis.[267] There is no clear-cut boundary between RRMS and SPMS, but rather a gradual shift characterized by the fact that the frequency of relapses subside while irreversible disability accrues despite pharmacological and rehabilitation interventions. Subjectively, patients transitioning from RRMS to SPMS report increased fatigue and decreased quality of life. [268] Several studies have identified risk factors for the conversion into a progressive forms, mostly represented by: age at onset >40, multifocal onset, high frequency of relapses, residual disability after relapses, and spinal cord involvement.[267,269] However, a biomarker for the transitional phase has not been identified yet and would be of

paramount importance, especially considering that currently approved disease modifying treatments are only moderately effective on reducing the gradual accrual of disability. One of the studies reported in this thesis presents a novel biomarker, named atrophied T2-lesion volume, a longitudinal measure representing T2 lesion volume progressively substituted by CSF. [270] The specific histological correlate of this marker has yet to be defined and is likely to be more than the mere reflection of the neuroinflammatory component related to focal lesions or the neurodegenerative process leading to tissue loss, as it explains additional variance in predicting disability even after controlling for both percentage brain volume change and enlarging/new lesions. [270,271] Furthermore, atrophied T2-lesion volume has a stronger association with clinical disability than new/enlarging lesions and brain atrophy, is correlated with global and thalamic volume measures and has been shown to be an early predictor of long-term disability progression. [270-273] With our study, while confirming the previous findings, we also demonstrated that atrophied T2-lesion volume was the only marker able to differentiate patients developing disease progression from stable ones, in the context of a multidisciplinary study applying novel imaging tools (e.g., OCT) and quantifying biomarkers strongly correlated with disease progression such as sNfL.[2] However, the small sample of PMS might have affected the ability to find correlations between atrophied T2-LV and other imaging measures in this subgroup of patients. Our findings were confirmed by a subsequent study that further characterized the nature of atrophied T2-LV by applying DTI imaging to a number of T2-lesions, dividing them according to whether they became atrophied T2 lesional tissue or not.[274] Interestingly, lesions destined to become atrophied T2-LV were all characterized by significantly altered DTI metrics, and especially by altered free-water, confirming the neurodegenerative component reflected by this MRI marker. As previously reported by our study, disease-progressed patients showed greater atrophied T2-LV. While further studies are needed to better elucidate the specific histological correlate of atrophied T2-lesion volume, this marker shows the potential to be applied in studies investigating the transitional phase of MS and, more generally, the phenomenon of disease progression, for its strong association with clinical and

MRI markers of disease evolution. The usefulness of atrophied T2-LV in clinical practice is currently hampered by the objective difficulty to visually assess lesional tissue changes over short periods of time. We are currently in the process of evaluating the feasibility of developing a visual scale to qualitatively assess atrophied T2-LV.

In parallel with the development of more sophisticated MRI techniques, new imaging modalities have been applied to the study of MS, such as OCT. Besides being non-invasive, inexpensive, well-tolerated and easy to use, this technique has also recently been confirmed to be highly reproducible, with an intraclass correlation coefficient ≥ 0.98. [275] A growing body of evidence in the last years has supported the use of OCT as a marker of retinal neuroaxonal degeneration and, thanks to its correlation with brain atrophy, of cerebral neurodegeneration as well. Indeed, OCT markers such as pRNFLT and macular GCIP (mGCIP) have been shown: to be significantly lower in MS vs. HC, as well as in RRMS vs. PMS; and to correlate with clinical disability, measures of cognitive impairment, and global and regional brain atrophy.[2,5,276-279] Our longitudinal study presented in this thesis confirmed and built upon previous findings, finding a strong significant correlation between OCT markers and sNfL, a biomarker of both neuroinflammation and neurodegeneration in MS patients. Whereas one previous study has investigated the association between sNfL and RNFLT in RRMS,[280] to the best our knowledge no other results have been reported before on the association between sNfL and several OCT markers, in different MS phenotypes.

Since publication of the study presented in this thesis, sNfL has been investigated by several studies for its association with the neurodegenerative component of MS and its easy accessibility.. Interestingly, in our study all the associations between sNfL and OCT markers were found in eyes not affected by optic neuritis. In the overall MS group sNfL was associated with pRNFLT at baseline and follow-up and mGCIP, in RRMS it was associated with both baseline pRNFLT and mGCIP, and in PMS there was a trending association between sNfL and mGCIP. These results support the role of all the analyzed markers in monitoring disease progression. One of the possible

explanations for the lack of significant associations in eyes affected by optic neuritis is that the focal damage due to past inflammatory episodes affects the ability of the OCT markers to reflect global neuroaxonal degeneration. Moreover, both pRNFLT and mGCIP were significantly decreased in the whole MS cohort, including both patients with and without a history of optic neuritis, possibly indicating a process of retinal neuroaxonal degeneration occurring, at least partially, independently from acute neuroinflammatory episodes. [281,282] Considering only the patients without a history of ON, the reduced OCT markers might be interpreted as the result of subclinical past ON attacks or as retrograde degeneration stemming from lesions along the optic pathways. [283,284]

The findings of our study were confirmed by several others in both cross-sectional and longitudinal studies, and in one population-based study on 4369 persons, of which 23 affected by MS. [285-289] Moreover, by supporting the role of neurodegenerative markers for both sNfL and OCT markers, our findings stimulated further analyses such as testing the association between OCT measures and scores of cognitive dysfunction, which strengthen the role of OCT markers in characterizing neurodegeneration. [290]

Advanced MRI techniques are useful not only to gain information on pathogenetic mechanisms, but also to monitor disease evolution or response to pharmacological and non-pharmacological treatments. Neuromotor rehabilitation has been integral to clinical practice for a long time. Our study, confirmed the benefits of neurorehabilitation, as demonstrated by the significant improvement on balance and gait function. Furthermore, it showed functional reorganization of the brain following the intensive neuromotor rehabilitation intervention, as we detected a reduced peak of activation in the precentral gyrus in response to the motor task, and an increased connectivity in the primary motor and somatosensory cortex with resting state fMRI. With respect to the former result, some studies have reported similar findings, with reduced activations of motor areas in response to motor rehabilitation, [291,292] but some others have described positive clinical outcomes paralleled by increased activation of cortical areas. [243,293-295]

The apparently contradictory findings with respect to changes in connectivity need to be interpreted taking into consideration positive adaptive brain plasticity vs. maladaptive plasticity. Adaptive plasticity reflects the brain's ability to structurally and functionally modify itself in response to experiences and environmental stimuli as well as to change subsequent to brain tissue damage, aiming at restoring homeostasis. [296] Maladaptive plasticity, instead, represents an aberrant modification leading to a poor clinical outcome. [297] The border between the two mechanisms is very thin as there is no MRI marker specific for either phenomenon, and in MS is further complicated by the presence of an underlying chronic tissue injury. Overall though, the association between reduced cortical activation and improved clinical measures lends itself to the interpretation of functional reorganization reflecting positive adaptive plasticity, driven by the attempt to "recover" the lost function. In particular, the reduced activation of the precentral gyrus found in our study in response to the motor task might be seen as an increased synaptic efficiency, resulting in a decreased energy demand to perform the same movement. This is indirectly supported by the increased global connectivity within primary motor and somatosensory cortex during resting state fMRI. Previously, increased functional connectivity has been described in response to cognitive training, [298] but no other study utilizing resting state fMRI after neuromotor rehabilitation in MS has been previously published. No structural changes were detected after the rehabilitative cycle, possibly due to the small sample size, and all the functional improvements found after the inpatient rehabilitation were no longer present three months later. The most likely explanation for the latter finding is that patients with medium-high disability do not maintain a level of physical activity sufficient to consolidate benefits in response to the intensive rehabilitation intervention If this interpretation were to be confirmed by longitudinal studies specifically investigating the level of physical activity at home for MS patients, rehabilitation should be available to patients in earlier stage of the disease, in which structural and functional brain resources are less limited and gains might be stronger and with longer-lasting effects. Second, patients with a higher level of disability should follow a different treatment path, in which intensive rehabilitation should be accompanied

by an appropriate training for home-based exercises, possibly with professional supervision and should be alternated with outpatient rehabilitative cycles aimed at consolidating the process of positive brain reorganization.

Our study has been included in reviews investigating the role of physical exercise on MRI markers and/or biological markers of CNS damage in MS, leading to the conclusion that exercise training has a positive role and should be recommended even in the early disease stage. However, evidence that neuroplastic mechanisms underpin benefits from rehabilitation is still incomplete and needs to be further investigated. [155,299-305]

The most common methodological flaws, hampering the robustness of results of neurorehabilitation studies, consist of small sample sizes, high drop-out rates, lack of proper randomization and blindness of patients/examiners when two treatments are compared and lack of a proper control group. Moreover, in our study we encountered some difficulties with the motor task while performing fMRI, as foot plantar dorsiflexion is a difficult task to perform and maintain over time, as the presence of muscle weakness, spasticity, and fatigability might interfere with the performance.

The findings of our combined studies highlight that MS disease progression cannot be properly characterized using conventional radiological imaging, or even a single advanced neuroimaging modality. Rather, the work presented in this thesis shows the utility of multimodal imaging techniques in leading to a better understanding of the underlying mechanisms of the disease. It is the hope that with such an approach, the puzzle of MS, particularly with respect to disease progression, can be further unraveled.

Future directions

Since the papers included in the current project have been published, several advances aimed at optimizing the techniques described here have been finalized, improving the sensitivity of diffusion imaging, by using multi-shell diffusion imaging, associating different susceptibility imaging approaches to clearly distinguish chemical components with different magnetic properties,

etc. Future technical developments of the already existing techniques might allow a more precise structural and functional characterization of the different tissue compartments, and a better understanding of the hierarchical organization of the brain hubs and networks.

Future studies on this topic will benefit from the availability of large population-based data registries including application of advanced imaging e.g. UK Biobank,[306] that facilitate association-based studies on a massive scale, overcoming one of the most common methodological limits that we have encountered in the studies reported here, the potential lack of significance due to small sample sizes. In the meantime, the optimization of advanced techniques and sequences will allow to obtain more detailed information at a microstructural, metabolic and functional level, allowing for a more in-depth knowledge on pathogenetic mechanisms active from early disease stages. This will also facilitate studies investigating the hypothesis that beneficial neuroplasticity underpins functional benefits from neuromotor rehabilitation.

Alternative imaging techniques such as OCT, as here reported, but also positron emission tomography (PET) are an active field of research as well, for their capability to provide complementary information on disease progression and measures of neuroinflammation.[307] Finally, machine learning approaches are being currently integrated in scientific research and will be extremely important for future studies in MS, as they will facilitate the diagnostic process, the identification of distinctive features of different disease stages and phenotypes, and the prediction of MS disease progression.

References

1. Zivadinov R, Tavazzi E, Bergsland N, et al. Brain Iron at Quantitative MRI Is Associated with Disability in Multiple Sclerosis. *Radiology* 2018; **289**(2): 487-96.

2. Tavazzi E, Bergsland N, Kuhle J, et al. A multimodal approach to assess the validity of atrophied T2-lesion volume as an MRI marker of disease progression in multiple sclerosis. *J Neurol* 2020; **267**(3): 802-11.

3. Tavazzi E, Jakimovski D, Kuhle J, et al. Serum neurofilament light chain and optical coherence tomography measures in MS: A longitudinal study. *Neurol Neuroimmunol Neuroinflamm* 2020; **7**(4).

4. Tavazzi E, Bergsland N, Cattaneo D, et al. Effects of motor rehabilitation on mobility and brain plasticity in multiple sclerosis: a structural and functional MRI study. *J Neurol* 2018; **265**(6): 1393-401.

5. Tavazzi E, Zivadinov R, Dwyer MG, et al. MRI biomarkers of disease progression and conversion to secondary-progressive multiple sclerosis. *Expert Rev Neurother* 2020; **20**(8): 821-34.

6. Bergsland N, Tavazzi E, Schweser F, et al. Targeting Iron Dyshomeostasis for Treatment of Neurodegenerative Disorders. *CNS Drugs* 2019; **33**(11): 1073-86.

7. Orrell R. Multiple Sclerosis: The History of a Disease. *Journal of the Royal Society of Medicine* 2005; **98**.

8. Zalc B. One hundred and fifty years ago Charcot reported multiple sclerosis as a new neurological disease. *Brain* 2018; **141**(12): 3482-8.

9. Curtius F SH. Multiple Sklerose und Erbanlage. *Zeitschrift für die gesamte Neurologie und Psychiatrie* 1938; **160**: 226-45.

10. Kabat EA, Moore DH, Landow H. An Electrophoretic Study of the Protein Components in Cerebrospinal Fluid and Their Relationship to the Serum Proteins. *J Clin Invest* 1942; **21**(5): 571-7.

11. Schumacher GA, Beebe G, Kibler RF, et al. Problems of Experimental Trials of Therapy in Multiple Sclerosis: Report by the Panel on the Evaluation of Experimental Trials of Therapy in Multiple Sclerosis. *Ann N Y Acad Sci* 1965; **122**: 552-68.

12. Poser CM, Paty DW, Scheinberg L, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983; **13**(3): 227-31.

13. Paty DW, Oger JJ, Kastrukoff LF, et al. MRI in the diagnosis of MS: a prospective study with comparison of clinical evaluation, evoked potentials, oligoclonal banding, and CT. *Neurology* 1988; **38**(2): 180-5.

14. Fazekas F, Offenbacher H, Fuchs S, et al. Criteria for an increased specificity of MRI interpretation in elderly subjects with suspected multiple sclerosis. *Neurology* 1988; **38**(12): 1822-5.

15. Barkhof F, Filippi M, Miller DH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. *Brain* 1997; **120 (Pt 11)**: 2059-69.

16. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol* 2001; **50**(1): 121-7.

17. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 2005; **58**(6): 840-6.

18. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011; **69**(2): 292-302.

19. Thompson AJ, Banwell BL, Barkhof F, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol* 2018; **17**(2): 162-73.

20. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group. *Neurology* 1993; **43**(4): 655-61.

21. Giovannoni G, Butzkueven H, Dhib-Jalbut S, et al. Brain health: time matters in multiple sclerosis. *Mult Scler Relat Disord* 2016; **9 Suppl 1**: S5-S48.

22. Goodin DS, Bates D. Treatment of early multiple sclerosis: the value of treatment initiation after a first clinical episode. *Mult Scler* 2009; **15**(10): 1175-82.

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

24. Reynders T, D'Haeseleer M, De Keyser J, Nagels G, D'Hooghe M B. Definition, prevalence and predictive factors of benign multiple sclerosis. *eNeurologicalSci* 2017; **7**: 37-43.

25. Ellenberger D, Flachenecker P, Haas J, et al. Is benign MS really benign? What a meaningful classification beyond the EDSS must take into consideration. *Mult Scler Relat Disord* 2020; **46**: 102485.

26. Hosseiny M, Newsome SD, Yousem DM. Radiologically Isolated Syndrome: A Review for Neuroradiologists. *AJNR Am J Neuroradiol* 2020; **41**(9): 1542-9.

27. Kurtzke JF, Beebe GW, Nagler B, Auth TL, Kurland LT, Nefzger MD. Studies on the natural history of multiple sclerosis. 6. Clinical and laboratory findings at first diagnosis. *Acta Neurol Scand* 1972; **48**(1): 19-46.

28. Kurtzke JF. A new scale for evaluating disability in multiple sclerosis. *Neurology* 1955; **5**(8): 580-3.

29. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* 1983; **33**(11): 1444-52.

30. Sharrack B, Hughes RA. Clinical scales for multiple sclerosis. *J Neurol Sci* 1996; **135**(1): 1-9.

31. Sipe JC, Knobler RL, Braheny SL, Rice GP, Panitch HS, Oldstone MB. A neurologic rating scale (NRS) for use in multiple sclerosis. *Neurology* 1984; **34**(10): 1368-72.

32. Fischer JS, Rudick RA, Cutter GR, Reingold SC. The Multiple Sclerosis Functional Composite Measure (MSFC): an integrated approach to MS clinical outcome assessment. National MS Society Clinical Outcomes Assessment Task Force. *Mult Scler* 1999; **5**(4): 244-50.

33. Roxburgh RH, Seaman SR, Masterman T, et al. Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity. *Neurology* 2005; **64**(7): 1144-51.

34. Collaborators GBDMS. Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* 2019; **18**(3): 269-85.

35. Collaborators GBDN. Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* 2019; **18**(5): 459-80.

36. Celius EG, Vandvik B. Multiple sclerosis in Oslo, Norway: prevalence on 1 January 1995 and incidence over a 25-year period. *Eur J Neurol* 2001; **8**(5): 463-9.

37. Simonsen CS, Edland A, Berg-Hansen P, Celius EG. High prevalence and increasing incidence of multiple sclerosis in the Norwegian county of Buskerud. *Acta Neurol Scand* 2017; **135**(4): 412-8.

38. Debouverie M, Pittion-Vouyovitch S, Louis S, Roederer T, Guillemin F. Increasing incidence of multiple sclerosis among women in Lorraine, Eastern France. *Mult Scler* 2007; **13**(8): 962-7.

39. Koch-Henriksen N, Thygesen LC, Stenager E, Laursen B, Magyari M. Incidence of MS has increased markedly over six decades in Denmark particularly with late onset and in women. *Neurology* 2018; **90**(22): e1954-e63.

40. Ahlgren C, Oden A, Lycke J. High nationwide incidence of multiple sclerosis in Sweden. *PLoS One* 2014; **9**(9): e108599.

41. Koch-Henriksen N, Magyari M. Apparent changes in the epidemiology and severity of multiple sclerosis. *Nat Rev Neurol* 2021; **17**(11): 676-88.

42. Adornato BT, Drogan O, Thoresen P, et al. The practice of neurology, 2000-2010: report of the AAN Member Research Subcommittee. *Neurology* 2011; **77**(21): 1921-8.

43. Kearns PKA, Paton M, O'Neill M, et al. Regional variation in the incidence rate and sex ratio of multiple sclerosis in Scotland 2010-2017: findings from the Scottish Multiple Sclerosis Register. *J Neurol* 2019; **266**(10): 2376-86.

44. Magyari M. Gender differences in multiple sclerosis epidemiology and treatment response. *Dan Med J* 2016; **63**(3).

45. Orton SM, Herrera BM, Yee IM, et al. Sex ratio of multiple sclerosis in Canada: a longitudinal study. *Lancet Neurol* 2006; **5**(11): 932-6.

46. Bostrom I, Stawiarz L, Landtblom AM. Sex ratio of multiple sclerosis in the National Swedish MS Register (SMSreg). *Mult Scler* 2013; **19**(1): 46-52.

47. McCombe PA, Greer JM. Female reproductive issues in multiple sclerosis. *Mult Scler* 2013; **19**(4): 392-402.

48. Del Boca D, Oggero N, Profeta P, Rossi M. Women's and men's work, housework and childcare, before and during COVID-19. *Rev Econ Househ* 2020: 1-17.

49. Ebers GC, Sadovnick AD. The geographic distribution of multiple sclerosis: a review. *Neuroepidemiology* 1993; **12**(1): 1-5.

50. Risco J, Maldonado H, Luna L, et al. Latitudinal prevalence gradient of multiple sclerosis in Latin America. *Mult Scler* 2011; **17**(9): 1055-9.

51. Simpson S, Jr., Blizzard L, Otahal P, Van der Mei I, Taylor B. Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis. *J Neurol Neurosurg Psychiatry* 2011; **82**(10): 1132-41.

52. Simpson S, Jr., Wang W, Otahal P, Blizzard L, van der Mei IAF, Taylor BV. Latitude continues to be significantly associated with the prevalence of multiple sclerosis: an updated meta-analysis. *J Neurol Neurosurg Psychiatry* 2019; **90**(11): 1193-200.

53. Lucas RM, Ponsonby AL, Dear K, et al. Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology* 2011; **76**(6): 540-8.

54. Simpson S, Jr., van der Mei I, Lucas RM, et al. Sun Exposure across the Life Course Significantly Modulates Early Multiple Sclerosis Clinical Course. *Front Neurol* 2018; **9**: 16.

55. Boltjes R, Knippenberg S, Gerlach O, Hupperts R, Damoiseaux J. Vitamin D supplementation in multiple sclerosis: an expert opinion based on the review of current evidence. *Expert Rev Neurother* 2021; **21**(6): 715-25.

56. Sabel CE, Pearson JF, Mason DF, Willoughby E, Abernethy DA, Taylor BV. The latitude gradient for multiple sclerosis prevalence is established in the early life course. *Brain* 2021; **144**(7): 2038-46.

57. Arneth B. Multiple Sclerosis and Smoking. *Am J Med* 2020; **133**(7): 783-8.

58. Chow HH, Talbot J, Marstrand L, et al. Smoking, cardiovascular risk factors and LRP2 gene variation: Associations with disease severity, cognitive function and brain structure in primary progressive multiple sclerosis. *Mult Scler Relat Disord* 2021; **56**: 103296.

59. Poorolajal J, Bahrami M, Karami M, Hooshmand E. Effect of smoking on multiple sclerosis: a meta-analysis. *J Public Health (Oxf)* 2017; **39**(2): 312-20.

60. Odoardi F, Sie C, Streyl K, et al. T cells become licensed in the lung to enter the central nervous system. *Nature* 2012; **488**(7413): 675-9.

61. Gao Z, Nissen JC, Ji K, Tsirka SE. The experimental autoimmune encephalomyelitis disease course is modulated by nicotine and other cigarette smoke components. *PLoS One* 2014; **9**(9): e107979.

62. Hernan MA, Jick SS, Logroscino G, Olek MJ, Ascherio A, Jick H. Cigarette smoking and the progression of multiple sclerosis. *Brain* 2005; **128**(Pt 6): 1461-5.

63. Manouchehrinia A, Tench CR, Maxted J, Bibani RH, Britton J, Constantinescu CS. Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study. *Brain* 2013; **136**(Pt 7): 2298-304.

64. Zivadinov R, Weinstock-Guttman B, Hashmi K, et al. Smoking is associated with increased lesion volumes and brain atrophy in multiple sclerosis. *Neurology* 2009; **73**(7): 504-10.

65. Ramanujam R, Hedstrom AK, Manouchehrinia A, et al. Effect of Smoking Cessation on Multiple Sclerosis Prognosis. *JAMA Neurol* 2015; **72**(10): 1117-23.

66. Rodgers J, Friede T, Vonberg FW, et al. The impact of smoking cessation on multiple sclerosis disease progression. *Brain* 2021.

67. Abbaszadeh S, Tabary M, Aryannejad A, et al. Air pollution and multiple sclerosis: a comprehensive review. *Neurol Sci* 2021; **42**(10): 4063-72.

68. Cortese A, Lova L, Comoli P, et al. Air pollution as a contributor to the inflammatory activity of multiple sclerosis. *J Neuroinflammation* 2020; **17**(1): 334.

69. Gregory AC, 2nd, Shendell DG, Okosun IS, Gieseker KE. Multiple Sclerosis disease distribution and potential impact of environmental air pollutants in Georgia. *Sci Total Environ* 2008; **396**(1): 42-51.

70. Turk Boru U, Boluk C, Tasdemir M, Gezer T, Serim VA. Air pollution, a possible risk factor for multiple sclerosis. *Acta Neurol Scand* 2020; **141**(5): 431-7.

71. Boru UT, Bilgic AB, Koseoglu Toksoy C, et al. Prevalence of Multiple Sclerosis in a Turkish City Bordering an Iron and Steel Factory. *J Clin Neurol* 2018; **14**(2): 234-41.

72. Oikonen M, Laaksonen M, Laippala P, et al. Ambient air quality and occurrence of multiple sclerosis relapse. *Neuroepidemiology* 2003; **22**(1): 95-9.

73. Roux J, Bard D, Le Pabic E, et al. Air pollution by particulate matter PM10 may trigger multiple sclerosis relapses. *Environ Res* 2017; **156**: 404-10.

74. Bergamaschi R, Cortese A, Pichiecchio A, et al. Air pollution is associated to the multiple sclerosis inflammatory activity as measured by brain MRI. *Mult Scler* 2018; **24**(12): 1578-84.

75. Bergamaschi R, Monti MC, Trivelli L, et al. PM2.5 exposure as a risk factor for multiple sclerosis. An ecological study with a Bayesian mapping approach. *Environ Sci Pollut Res Int* 2021; **28**(3): 2804-9.

76. Tateo F, Grassivaro F, Ermani M, Puthenparampil M, Gallo P. PM2.5 levels strongly associate with multiple sclerosis prevalence in the Province of Padua, Veneto Region, North-East Italy. *Mult Scler* 2019; **25**(13): 1719-27.

77. Bergamaschi R, Montomoli C. Air pollution is a risk factor for multiple sclerosis - Yes. *Mult Scler* 2021; **27**(14): 2137-8.

78. Delgado-Garcia G, Corona T. Air pollution is a risk factor for multiple sclerosis - No. *Mult Scler* 2021; **27**(14): 2138-9.

79. Fernandez-Menendez S, Fernandez-Moran M, Fernandez-Vega I, Perez-Alvarez A, Villafani-Echazu J. Epstein-Barr virus and multiple sclerosis. From evidence to therapeutic strategies. *J Neurol Sci* 2016; **361**: 213-9.

80. Pender MP, Burrows SR. Epstein-Barr virus and multiple sclerosis: potential opportunities for immunotherapy. *Clin Transl Immunology* 2014; **3**(10): e27.

81. Ascherio A, Munger KL. Epstein-barr virus infection and multiple sclerosis: a review. *J Neuroimmune Pharmacol* 2010; **5**(3): 271-7.

82. Bar-Or A, Pender MP, Khanna R, et al. Epstein-Barr Virus in Multiple Sclerosis: Theory and Emerging Immunotherapies. *Trends Mol Med* 2020; **26**(3): 296-310.

83. Bjornevik K, Cortese M, Healy BC, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science* 2022; **375**(6578): 296-301.

84. Houen G, Trier NH, Frederiksen JL. Epstein-Barr Virus and Multiple Sclerosis. *Front Immunol* 2020; **11**: 587078.

85. Esposito S, Bonavita S, Sparaco M, Gallo A, Tedeschi G. The role of diet in multiple sclerosis: A review. *Nutr Neurosci* 2018; **21**(6): 377-90.

86. Ochoa-Reparaz J, Kirby TO, Kasper LH. The Gut Microbiome and Multiple Sclerosis. *Cold Spring Harb Perspect Med* 2018; **8**(6).

87. Munger KL, Chitnis T, Ascherio A. Body size and risk of MS in two cohorts of US women. *Neurology* 2009; **73**(19): 1543-50.

88. Munger KL, Bentzen J, Laursen B, et al. Childhood body mass index and multiple sclerosis risk: a long-term cohort study. *Mult Scler* 2013; **19**(10): 1323-9.

89. Hedstrom AK, Olsson T, Alfredsson L. High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women. *Mult Scler* 2012; **18**(9): 1334-6.

90. Esposito K, Pontillo A, Di Palo C, et al. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. *JAMA* 2003; **289**(14): 1799-804.

91. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Low-grade systemic inflammation in overweight children. *Pediatrics* 2001; **107**(1): E13.

92. Timpson NJ, Nordestgaard BG, Harbord RM, et al. C-reactive protein levels and body mass index: elucidating direction of causation through reciprocal Mendelian randomization. *Int J Obes (Lond)* 2011; **35**(2): 300-8.

93. Ebers GC. Environmental factors and multiple sclerosis. *Lancet Neurol* 2008; **7**(3): 268-77.

94. Fitzgerald KC, Tyry T, Salter A, et al. Diet quality is associated with disability and symptom severity in multiple sclerosis. *Neurology* 2018; **90**(1): e1-e11.

95. Katz Sand I. The Role of Diet in Multiple Sclerosis: Mechanistic Connections and Current Evidence. *Curr Nutr Rep* 2018; **7**(3): 150-60.

96. Bjornevik K, Chitnis T, Ascherio A, Munger KL. Polyunsaturated fatty acids and the risk of multiple sclerosis. *Mult Scler* 2017; **23**(14): 1830-8.

97. Weinstock-Guttman B, Baier M, Park Y, et al. Low fat dietary intervention with omega-3 fatty acid supplementation in multiple sclerosis patients. *Prostaglandins Leukot Essent Fatty Acids* 2005; **73**(5): 397-404.

98. Bates D, Fawcett PR, Shaw DA, Weightman D. Polyunsaturated fatty acids in treatment of acute remitting multiple sclerosis. *Br Med J* 1978; **2**(6149): 1390-1.

99. Bates D, Fawcett PR, Shaw DA, Weightman D. Trial of polyunsaturated fatty acids in non-relapsing multiple sclerosis. *Br Med J* 1977; **2**(6092): 932-3.

100. Harbige LS, Sharief MK. Polyunsaturated fatty acids in the pathogenesis and treatment of multiple sclerosis. *Br J Nutr* 2007; **98 Suppl 1**: S46-53.

101. Choi IY, Piccio L, Childress P, et al. A Diet Mimicking Fasting Promotes Regeneration and Reduces Autoimmunity and Multiple Sclerosis Symptoms. *Cell Rep* 2016; **15**(10): 2136-46.

102. Bock M, Steffen F, Zipp F, Bittner S. Impact of Dietary Intervention on Serum Neurofilament Light Chain in Multiple Sclerosis. *Neurol Neuroimmunol Neuroinflamm* 2022; **9**(1).

103. Parks NE, Jackson-Tarlton CS, Vacchi L, Merdad R, Johnston BC. Dietary interventions for multiple sclerosis-related outcomes. *Cochrane Database Syst Rev* 2020; **5**: CD004192.

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

105. Tobin WO, Kalinowska-Lyszczarz A, Weigand SD, et al. Clinical Correlation of Multiple Sclerosis Immunopathologic Subtypes. *Neurology* 2021; **97**(19): e1906-e13.

106. Breij EC, Brink BP, Veerhuis R, et al. Homogeneity of active demyelinating lesions in established multiple sclerosis. *Ann Neurol* 2008; **63**(1): 16-25.

107. Lassmann H, Bruck W, Lucchinetti C, Rodriguez M. Remyelination in multiple sclerosis. *Mult Scler* 1997; **3**(2): 133-6.

108. Yeung MSY, Djelloul M, Steiner E, et al. Dynamics of oligodendrocyte generation in multiple sclerosis. *Nature* 2019; **566**(7745): 538-42.

109. Lee DH. Mechanisms of contrast enhancement in magnetic resonance imaging. *Can Assoc Radiol J* 1991; **42**(1): 6-12.

110. Klawiter EC. Current and new directions in MRI in multiple sclerosis. *Continuum (Minneap Minn)* 2013; **19**(4 Multiple Sclerosis): 1058-73.

111. Rocca MA, Comi G, Filippi M. The Role of T1-Weighted Derived Measures of Neurodegeneration for Assessing Disability Progression in Multiple Sclerosis. *Front Neurol* 2017; **8**: 433.

112. Chard D, Trip SA. Resolving the clinico-radiological paradox in multiple sclerosis. *F1000Res* 2017; **6**: 1828.

113. Bodini B, Battaglini M, De Stefano N, et al. T2 lesion location really matters: a 10 year follow-up study in primary progressive multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2011; **82**(1): 72-7.

114. Elliott C, Wolinsky JS, Hauser SL, et al. Slowly expanding/evolving lesions as a magnetic resonance imaging marker of chronic active multiple sclerosis lesions. *Mult Scler* 2019; **25**(14): 1915-25.

115. Elliott C, Arnold DL, Chen H, et al. Patterning Chronic Active Demyelination in Slowly Expanding/Evolving White Matter MS Lesions. *AJNR Am J Neuroradiol* 2020; **41**(9): 1584-91.

116. Elliott C, Belachew S, Wolinsky JS, et al. Chronic white matter lesion activity predicts clinical progression in primary progressive multiple sclerosis. *Brain* 2019; **142**(9): 2787-99.

117. Pinto C, Cambron M, Dobai A, Vanheule E, Casselman JW. Smoldering lesions in MS: if you like it then you should put a rim on it. *Neuroradiology* 2021.

118. Moll NM, Rietsch AM, Thomas S, et al. Multiple sclerosis normal-appearing white matter: pathology-imaging correlations. *Ann Neurol* 2011; **70**(5): 764-73.

119. Allen IV, McQuaid S, Mirakhur M, Nevin G. Pathological abnormalities in the normal-appearing white matter in multiple sclerosis. *Neurol Sci* 2001; **22**(2): 141-4.

120. Evangelou N, Konz D, Esiri MM, Smith S, Palace J, Matthews PM. Regional axonal loss in the corpus callosum correlates with cerebral white matter lesion volume and distribution in multiple sclerosis. *Brain* 2000; **123 (Pt 9)**: 1845-9.

121. Evangelou N, Konz D, Esiri MM, Smith S, Palace J, Matthews PM. Size-selective neuronal changes in the anterior optic pathways suggest a differential susceptibility to injury in multiple sclerosis. *Brain* 2001; **124**(Pt 9): 1813-20.

122. Peterson JW, Bo L, Mork S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Ann Neurol* 2001; **50**(3): 389-400.

123. Lassmann H. Multiple Sclerosis Pathology. *Cold Spring Harb Perspect Med* 2018; **8**(3).

124. Bo L, Vedeler CA, Nyland HI, Trapp BD, Mork SJ. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. *J Neuropathol Exp Neurol* 2003; **62**(7): 723-32.

125. Kidd D, Barkhof F, McConnell R, Algra PR, Allen IV, Revesz T. Cortical lesions in multiple sclerosis. *Brain* 1999; **122 (Pt 1)**: 17-26.

126. Calabrese M, Rocca MA, Atzori M, et al. A 3-year magnetic resonance imaging study of cortical lesions in relapse-onset multiple sclerosis. *Ann Neurol* 2010; **67**(3): 376-83.

127. Madsen MAJ, Wiggermann V, Bramow S, Christensen JR, Sellebjerg F, Siebner HR. Imaging cortical multiple sclerosis lesions with ultra-high field MRI. *Neuroimage Clin* 2021; **32**: 102847.

128. Seewann A, Vrenken H, Kooi EJ, et al. Imaging the tip of the iceberg: visualization of cortical lesions in multiple sclerosis. *Mult Scler* 2011; **17**(10): 1202-10.

129. Rinaldi F, Calabrese M, Grossi P, Puthenparampil M, Perini P, Gallo P. Cortical lesions and cognitive impairment in multiple sclerosis. *Neurol Sci* 2010; **31**(Suppl 2): S235-7.

130. Magliozzi R, Howell O, Vora A, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 2007; **130**(Pt 4): 1089-104.

131. Sen MK, Almuslehi MSM, Shortland PJ, Coorssen JR, Mahns DA. Revisiting the Pathoetiology of Multiple Sclerosis: Has the Tail Been Wagging the Mouse? *Front Immunol* 2020; **11**: 572186.

132. Sandi D, Fricska-Nagy Z, Bencsik K, Vecsei L. Neurodegeneration in Multiple Sclerosis: Symptoms of Silent Progression, Biomarkers and Neuroprotective Therapy-Kynurenines Are Important Players. *Molecules* 2021; **26**(11).

133. Frischer JM, Bramow S, Dal-Bianco A, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* 2009; **132**(Pt 5): 1175-89.

134. Benedict RH, Bruce JM, Dwyer MG, et al. Neocortical atrophy, third ventricular width, and cognitive dysfunction in multiple sclerosis. *Arch Neurol* 2006; **63**(9): 1301-6.

135. Bermel RA, Bakshi R, Tjoa C, Puli SR, Jacobs L. Bicaudate ratio as a magnetic resonance imaging marker of brain atrophy in multiple sclerosis. *Arch Neurol* 2002; **59**(2): 275-80.

136. Rudick RA, Fisher E, Lee JC, Duda JT, Simon J. Brain atrophy in relapsing multiple sclerosis: relationship to relapses, EDSS, and treatment with interferon beta-1a. *Mult Scler* 2000; **6**(6): 365-72.

137. Smith SM, De Stefano N, Jenkinson M, Matthews PM. Normalized accurate measurement of longitudinal brain change. *J Comput Assist Tomogr* 2001; **25**(3): 466-75.

138. Jacobsen C, Zivadinov R, Myhr KM, et al. Brain atrophy and clinical characteristics predicting SDMT performance in multiple sclerosis: A 10-year follow-up study. *Mult Scler J Exp Transl Clin* 2021; **7**(1): 2055217321992394.

139. Jacobsen C, Zivadinov R, Myhr KM, et al. Brain atrophy and employment in multiple sclerosis patients: a 10-year follow-up study. *Mult Scler J Exp Transl Clin* 2020; **6**(1): 2055217320902481.

140. Zivadinov R, Uher T, Hagemeier J, et al. A serial 10-year follow-up study of brain atrophy and disability progression in RRMS patients. *Mult Scler* 2016; **22**(13): 1709-18.

141. Bergsland N, Zivadinov R, Dwyer MG, Weinstock-Guttman B, Benedict RH. Localized atrophy of the thalamus and slowed cognitive processing speed in MS patients. *Mult Scler* 2016; **22**(10): 1327-36.

142. Hanninen K, Viitala M, Paavilainen T, et al. Thalamic Atrophy Predicts 5-Year Disability Progression in Multiple Sclerosis. *Front Neurol* 2020; **11**: 606.

143. Zivadinov R, Jakimovski D, Gandhi S, et al. Clinical relevance of brain atrophy assessment in multiple sclerosis. Implications for its use in a clinical routine. *Expert Rev Neurother* 2016; **16**(7): 777-93.

144. Kappos L, De Stefano N, Freedman MS, et al. Inclusion of brain volume loss in a revised measure of 'no evidence of disease activity' (NEDA-4) in relapsing-remitting multiple sclerosis. *Mult Scler* 2016; **22**(10): 1297-305.

145. Uher T, Bergsland N, Krasensky J, et al. Interpretation of Brain Volume Increase in Multiple Sclerosis. *J Neuroimaging* 2021; **31**(2): 401-7.

146. De Stefano N, Stromillo ML, Giorgio A, et al. Establishing pathological cut-offs of brain atrophy rates in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2016; **87**(1): 93-9.

147. Bergsland N, Lagana MM, Tavazzi E, et al. Corticospinal tract integrity is related to primary motor cortex thinning in relapsing-remitting multiple sclerosis. *Mult Scler* 2015; **21**(14): 1771-80.

148. Bammer R, Augustin M, Strasser-Fuchs S, et al. Magnetic resonance diffusion tensor imaging for characterizing diffuse and focal white matter abnormalities in multiple sclerosis. *Magn Reson Med* 2000; **44**(4): 583-91.

149. Filippi M, Cercignani M, Inglese M, Horsfield MA, Comi G. Diffusion tensor magnetic resonance imaging in multiple sclerosis. *Neurology* 2001; **56**(3): 304-11.

150. Filippi M, Iannucci G, Cercignani M, Assunta Rocca M, Pratesi A, Comi G. A quantitative study of water diffusion in multiple sclerosis lesions and normal-appearing white matter using echo-planar imaging. *Arch Neurol* 2000; **57**(7): 1017-21.

151. Daams M, Steenwijk MD, Wattjes MP, et al. Unraveling the neuroimaging predictors for motor dysfunction in long-standing multiple sclerosis. *Neurology* 2015; **85**(3): 248-55.

152. Steenwijk MD, Geurts JJ, Daams M, et al. Cortical atrophy patterns in multiple sclerosis are non-random and clinically relevant. *Brain* 2016; **139**(Pt 1): 115-26.

153. Steenwijk MD, Daams M, Pouwels PJ, et al. Unraveling the relationship between regional gray matter atrophy and pathology in connected white matter tracts in long-standing multiple sclerosis. *Hum Brain Mapp* 2015; **36**(5): 1796-807.

154. Brown GG, Perthen JE, Liu TT, Buxton RB. A primer on functional magnetic resonance imaging. *Neuropsychol Rev* 2007; **17**(2): 107-25.

155. Tavazzi E, Cazzoli M, Pirastru A, et al. Neuroplasticity and Motor Rehabilitation in Multiple Sclerosis: A Systematic Review on MRI Markers of Functional and Structural Changes. *Front Neurosci* 2021; **15**: 707675.

156. Rocca MA, Colombo B, Falini A, et al. Cortical adaptation in patients with MS: a cross-sectional functional MRI study of disease phenotypes. *Lancet Neurol* 2005; **4**(10): 618-26.

157. Damoiseaux JS, Rombouts SA, Barkhof F, et al. Consistent resting-state networks across healthy subjects. *Proc Natl Acad Sci U S A* 2006; **103**(37): 13848-53.

158. Dipasquale O, Cercignani M. Network functional connectivity and whole-brain functional connectomics to investigate cognitive decline in neurodegenerative conditions. *Funct Neurol* 2016; **31**(4): 191-203.

159. Schoonheim MM. Functional reorganization is a maladaptive response to injury - Commentary. *Mult Scler* 2017; **23**(2): 194-6.

160. Rocca MA, Filippi M. Functional reorganization is a maladaptive response to injury - YES. *Mult Scler* 2017; **23**(2): 191-3.

161. Hare D, Ayton S, Bush A, Lei P. A delicate balance: Iron metabolism and diseases of the brain. *Front Aging Neurosci* 2013; **5**: 34.

162. Horowitz MP, Greenamyre JT. Mitochondrial iron metabolism and its role in neurodegeneration. *J Alzheimers Dis* 2010; **20 Suppl 2**: S551-68.

163. Zhang C. Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control. *Protein Cell* 2014; **5**(10): 750-60.

164. Bakshi R, Benedict RH, Bermel RA, et al. T2 hypointensity in the deep gray matter of patients with multiple sclerosis: a quantitative magnetic resonance imaging study. *Arch Neurol* 2002; **59**(1): 62-8.

165. Tjoa CW, Benedict RH, Weinstock-Guttman B, Fabiano AJ, Bakshi R. MRI T2 hypointensity of the dentate nucleus is related to ambulatory impairment in multiple sclerosis. *J Neurol Sci* 2005; **234**(1-2): 17-24.

166. Bartzokis G, Beckson M, Hance DB, Marx P, Foster JA, Marder SR. MR evaluation of age-related increase of brain iron in young adult and older normal males. *Magn Reson Imaging* 1997; **15**(1): 29-35.

167. Bergsland N, Agostini S, Lagana MM, et al. Serum iron concentration is associated with subcortical deep gray matter iron levels in multiple sclerosis patients. *Neuroreport* 2017; **28**(11): 645-8.

168. Hagemeier J, Zivadinov R, Dwyer MG, et al. Changes of deep gray matter magnetic susceptibility over 2 years in multiple sclerosis and healthy control brain. *Neuroimage Clin* 2018; **18**: 1007-16.

169. Fujiwara E, Kmech JA, Cobzas D, et al. Cognitive Implications of Deep Gray Matter Iron in Multiple Sclerosis. *AJNR Am J Neuroradiol* 2017; **38**(5): 942-8.

170. Schweser F, Hagemeier J, Dwyer MG, et al. Decreasing brain iron in multiple sclerosis: The difference between concentration and content in iron MRI. *Hum Brain Mapp* 2021; **42**(5): 1463-74.

171. Absinta M, Maric D, Gharagozloo M, et al. A lymphocyte-microglia-astrocyte axis in chronic active multiple sclerosis. *Nature* 2021; **597**(7878): 709-14.

172. Filippi M, Rocca MA, Martino G, Horsfield MA, Comi G. Magnetization transfer changes in the normal appearing white matter precede the appearance of enhancing lesions in patients with multiple sclerosis. *Ann Neurol* 1998; **43**(6): 809-14.

173. Filippi M. Magnetization transfer imaging to monitor the evolution of multiple sclerosis. *Ital J Neurol Sci* 1999; **20**(5 Suppl): S232-40.

174. Chou IJ, Lim SY, Tanasescu R, et al. Seven-Tesla Magnetization Transfer Imaging to Detect Multiple Sclerosis White Matter Lesions. *J Neuroimaging* 2018; **28**(2): 183-90.

175. Chen JT, Collins DL, Atkins HL, Freedman MS, Arnold DL, Canadian MSBMTSG. Magnetization transfer ratio evolution with demyelination and remyelination in multiple sclerosis lesions. *Ann Neurol* 2008; **63**(2): 254-62.

176. Mehmood A, Ali W, Song S, et al. Optical coherence tomography monitoring and diagnosing retinal changes in multiple sclerosis. *Brain Behav* 2021; **11**(10): e2302.

177. Gordon-Lipkin E, Calabresi PA. Optical coherence tomography: A quantitative tool to measure neurodegeneration and facilitate testing of novel treatments for tissue protection in multiple sclerosis. *J Neuroimmunol* 2017; **304**: 93-6.

178. Albrecht P, Frohlich R, Hartung HP, Kieseier BC, Methner A. Optical coherence tomography measures axonal loss in multiple sclerosis independently of optic neuritis. *J Neurol* 2007; **254**(11): 1595-6.

179. Burkholder BM, Osborne B, Loguidice MJ, et al. Macular volume determined by optical coherence tomography as a measure of neuronal loss in multiple sclerosis. *Arch Neurol* 2009; **66**(11): 1366-72.

180. Toledo J, Sepulcre J, Salinas-Alaman A, et al. Retinal nerve fiber layer atrophy is associated with physical and cognitive disability in multiple sclerosis. *Mult Scler* 2008; **14**(7): 906-12.

181. Garcia-Martin E, Rodriguez-Mena D, Herrero R, et al. Neuro-ophthalmologic evaluation, quality of life, and functional disability in patients with MS. *Neurology* 2013; **81**(1): 76-83.

182. Martinez-Lapiscina EH, Arnow S, Wilson JA, et al. Retinal thickness measured with optical coherence tomography and risk of disability worsening in multiple sclerosis: a cohort study. *Lancet Neurol* 2016; **15**(6): 574-84.

183. Dreyer-Alster S, Gal A, Achiron A. Optical Coherence Tomography Is Associated With Cognitive Impairment in Multiple Sclerosis. *J Neuroophthalmol* 2021.

184. Oertel FC, Specovius S, Zimmermann HG, et al. Retinal Optical Coherence Tomography in Neuromyelitis Optica. *Neurol Neuroimmunol Neuroinflamm* 2021; **8**(6).

185. Pittock SJ, Zekeridou A, Weinshenker BG. Hope for patients with neuromyelitis optica spectrum disorders - from mechanisms to trials. *Nat Rev Neurol* 2021; **17**(12): 759-73.

186. Takai Y, Misu T, Kaneko K, et al. Myelin oligodendrocyte glycoprotein antibody-associated disease: an immunopathological study. *Brain* 2020; **143**(5): 1431-46.

187. Correale J, de los Milagros Bassani Molinas M. Oligoclonal bands and antibody responses in multiple sclerosis. *J Neurol* 2002; **249**(4): 375-89.

188. Matute-Blanch C, Villar LM, Alvarez-Cermeno JC, et al. Neurofilament light chain and oligoclonal bands are prognostic biomarkers in radiologically isolated syndrome. *Brain* 2018; **141**(4): 1085-93.

189. Lycke JN, Karlsson JE, Andersen O, Rosengren LE. Neurofilament protein in cerebrospinal fluid: a potential marker of activity in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1998; **64**(3): 402-4.

190. Silber E, Semra YK, Gregson NA, Sharief MK. Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. *Neurology* 2002; **58**(9): 1372-81.

191. Van der Zee CE, Nielander HB, Vos JP, et al. Expression of growth-associated protein B-50 (GAP43) in dorsal root ganglia and sciatic nerve during regenerative sprouting. *J Neurosci* 1989; **9**(10): 3505-12.

192. Disanto G, Barro C, Benkert P, et al. Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. *Ann Neurol* 2017; **81**(6): 857-70.

193. Teunissen CE, Dijkstra C, Polman C. Biological markers in CSF and blood for axonal degeneration in multiple sclerosis. *Lancet Neurol* 2005; **4**(1): 32-41.

194. Bergman J, Dring A, Zetterberg H, et al. Neurofilament light in CSF and serum is a sensitive marker for axonal white matter injury in MS. *Neurol Neuroimmunol Neuroinflamm* 2016; **3**(5): e271.

195. Novakova L, Zetterberg H, Sundstrom P, et al. Monitoring disease activity in multiple sclerosis using serum neurofilament light protein. *Neurology* 2017; **89**(22): 2230-7.

196. Kuhle J, Nourbakhsh B, Grant D, et al. Serum neurofilament is associated with progression of brain atrophy and disability in early MS. *Neurology* 2017; **88**(9): 826-31.

197. Srpova B, Uher T, Hrnciarova T, et al. Serum neurofilament light chain reflects inflammation-driven neurodegeneration and predicts delayed brain volume loss in early stage of multiple sclerosis. *Mult Scler* 2021; **27**(1): 52-60.

198. Kuhle J, Kropshofer H, Haering DA, et al. Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. *Neurology* 2019; **92**(10): e1007-e15.

199. Barro C, Benkert P, Disanto G, et al. Serum neurofilament as a predictor of disease worsening and brain and spinal cord atrophy in multiple sclerosis. *Brain* 2018; **141**(8): 2382-91.

200. Serum neurofilament light chain levels associations with gray matter pathology: a 5-year longitudinal study. *Ann Clin Transl Neurol* 2019; **6**(12): 2607.

201. Chitnis T, Gonzalez C, Healy BC, et al. Neurofilament light chain serum levels correlate with 10-year MRI outcomes in multiple sclerosis. *Ann Clin Transl Neurol* 2018; **5**(12): 1478-91.

202. Piehl F, Kockum I, Khademi M, et al. Plasma neurofilament light chain levels in patients with MS switching from injectable therapies to fingolimod. *Mult Scler* 2018; **24**(8): 1046-54.

203. Delcoigne B, Manouchehrinia A, Barro C, et al. Blood neurofilament light levels segregate treatment effects in multiple sclerosis. *Neurology* 2020; **94**(11): e1201-e12.

204. Bittner S, Oh J, Havrdova EK, Tintore M, Zipp F. The potential of serum neurofilament as biomarker for multiple sclerosis. *Brain* 2021.

205. Cohan SL, Hendin BA, Reder AT, et al. Interferons and Multiple Sclerosis: Lessons from 25 Years of Clinical and Real-World Experience with Intramuscular Interferon Beta-1a (Avonex). *CNS Drugs* 2021; **35**(7): 743-67.

206. Kappos L, Gold R, Miller DH, et al. Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study. *Lancet* 2008; **372**(9648): 1463-72.

207. Wilms H, Sievers J, Rickert U, Rostami-Yazdi M, Mrowietz U, Lucius R. Dimethylfumarate inhibits microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1beta, TNF-alpha and IL-6 in an in-vitro model of brain inflammation. *J Neuroinflammation* 2010; **7**: 30.

208. Chun J, Hartung HP. Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. *Clin Neuropharmacol* 2010; **33**(2): 91-101.

209. Cohen JA, Barkhof F, Comi G, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N Engl J Med* 2010; **362**(5): 402-15.

210. Arvin AM, Wolinsky JS, Kappos L, et al. Varicella-zoster virus infections in patients treated with fingolimod: risk assessment and consensus recommendations for management. *JAMA Neurol* 2015; **72**(1): 31-9.

211. Sriwastava S, Chaudhary D, Srivastava S, et al. Progressive multifocal leukoencephalopathy and sphingosine 1-phosphate receptor modulators used in multiple sclerosis: an updated review of literature. *J Neurol* 2021.

212. Oh J, O'Connor PW. Teriflunomide in the treatment of multiple sclerosis: current evidence and future prospects. *Ther Adv Neurol Disord* 2014; **7**(5): 239-52.

213. O'Connor P, Wolinsky JS, Confavreux C, et al. Randomized trial of oral teriflunomide for relapsing multiple sclerosis. *N Engl J Med* 2011; **365**(14): 1293-303.

214. Giovannoni G, Comi G, Cook S, et al. A placebo-controlled trial of oral cladribine for relapsing multiple sclerosis. *N Engl J Med* 2010; **362**(5): 416-26.

215. Hutchinson M. Natalizumab: A new treatment for relapsing remitting multiple sclerosis. *Ther Clin Risk Manag* 2007; **3**(2): 259-68.

216. Miller DH, Khan OA, Sheremata WA, et al. A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 2003; **348**(1): 15-23.

217. Kleinschmidt-DeMasters BK, Tyler KL. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 2005; **353**(4): 369-74.

218. Hauser SL, Bar-Or A, Comi G, et al. Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med* 2017; **376**(3): 221-34.

219. Montalban X, Hauser SL, Kappos L, et al. Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *N Engl J Med* 2017; **376**(3): 209-20.

220. Chisari CG, Sgarlata E, Arena S, Toscano S, Luca M, Patti F. Rituximab for the treatment of multiple sclerosis: a review. *J Neurol* 2021.

221. Havrdova E, Horakova D, Kovarova I. Alemtuzumab in the treatment of multiple sclerosis: key clinical trial results and considerations for use. *Ther Adv Neurol Disord* 2015; **8**(1): 31-45.

222. Russo CV, Sacca F, Frau J, et al. A real-world study of alemtuzumab in a cohort of Italian patients. *Eur J Neurol* 2021.

223. Sormani MP, Salvetti M, Labauge P, et al. DMTs and Covid-19 severity in MS: a pooled analysis from Italy and France. *Ann Clin Transl Neurol* 2021; **8**(8): 1738-44.

224. Simpson-Yap S, De Brouwer E, Kalincik T, et al. Associations of Disease-Modifying Therapies With COVID-19 Severity in Multiple Sclerosis. *Neurology* 2021; **97**(19): e1870-e85.

225. Tortorella C, Aiello A, Gasperini C, et al. Humoral- and T-Cell-Specific Immune Responses to SARS-CoV-2 mRNA Vaccination in Patients With MS Using Different Disease-Modifying Therapies. *Neurology* 2021.

226. Garthe A, Roeder I, Kempermann G. Mice in an enriched environment learn more flexibly because of adult hippocampal neurogenesis. *Hippocampus* 2016; **26**(2): 261-71.

227. Lambert K, Eisch AJ, Galea LAM, Kempermann G, Merzenich M. Optimizing brain performance: Identifying mechanisms of adaptive neurobiological plasticity. *Neurosci Biobehav Rev* 2019; **105**: 60-71.

228. Maguire EA, Gadian DG, Johnsrude IS, et al. Navigation-related structural change in the hippocampi of taxi drivers. *Proc Natl Acad Sci U S A* 2000; **97**(8): 4398-403.

229. Zatorre RJ, Fields RD, Johansen-Berg H. Plasticity in gray and white: neuroimaging changes in brain structure during learning. *Nat Neurosci* 2012; **15**(4): 528-36.

230. Bengtsson SL, Nagy Z, Skare S, Forsman L, Forssberg H, Ullen F. Extensive piano practicing has regionally specific effects on white matter development. *Nat Neurosci* 2005; **8**(9): 1148-50.

231. Draganski B, Gaser C, Busch V, Schuierer G, Bogdahn U, May A. Neuroplasticity: changes in grey matter induced by training. *Nature* 2004; **427**(6972): 311-2.

232. Rossi S, Furlan R, De Chiara V, et al. Exercise attenuates the clinical, synaptic and dendritic abnormalities of experimental autoimmune encephalomyelitis. *Neurobiol Dis* 2009; **36**(1): 51-9.

233. Dalgas U, Stenager E, Jakobsen J, et al. Resistance training improves muscle strength and functional capacity in multiple sclerosis. *Neurology* 2009; **73**(18): 1478-84.

234. Guo LY, Lozinski B, Yong VW. Exercise in multiple sclerosis and its models: Focus on the central nervous system outcomes. *J Neurosci Res* 2020; **98**(3): 509-23.

235. Latimer-Cheung AE, Martin Ginis KA, Hicks AL, et al. Development of evidence-informed physical activity guidelines for adults with multiple sclerosis. *Arch Phys Med Rehabil* 2013; **94**(9): 1829-36 e7.

236. Lozinski BM, Yong VW. Exercise and the brain in multiple sclerosis. *Mult Scler* 2020: 1352458520969099.

237. El-Sayes J, Harasym D, Turco CV, Locke MB, Nelson AJ. Exercise-Induced Neuroplasticity: A Mechanistic Model and Prospects for Promoting Plasticity. *Neuroscientist* 2019; **25**(1): 65-85.

238. Motl RW, Pilutti LA, Hubbard EA, Wetter NC, Sosnoff JJ, Sutton BP. Cardiorespiratory fitness and its association with thalamic, hippocampal, and basal ganglia volumes in multiple sclerosis. *Neuroimage Clin* 2015; **7**: 661-6.

239. Prakash RS, Patterson B, Janssen A, Abduljalil A, Boster A. Physical activity associated with increased resting-state functional connectivity in multiple sclerosis. *J Int Neuropsychol Soc* 2011; **17**(6): 986-97.

240. Prakash RS, Snook EM, Erickson KI, et al. Cardiorespiratory fitness: A predictor of cortical plasticity in multiple sclerosis. *Neuroimage* 2007; **34**(3): 1238-44.

241. Kalb R, Brown TR, Coote S, et al. Exercise and lifestyle physical activity recommendations for people with multiple sclerosis throughout the disease course. *Mult Scler* 2020; **26**(12): 1459-69.

242. Koudriavtseva T, Mainero C. Neuroinflammation, neurodegeneration and regeneration in multiple sclerosis: intercorrelated manifestations of the immune response. *Neural Regen Res* 2016; **11**(11): 1727-30.

243. Rocca MA, Meani A, Fumagalli S, et al. Functional and structural plasticity following action observation training in multiple sclerosis. *Mult Scler* 2019; **25**(11): 1472-87.

244. Rasova K, Prochazkova M, Tintera J, Ibrahim I, Zimova D, Stetkarova I. Motor programme activating therapy influences adaptive brain functions in multiple sclerosis: clinical and MRI study. *Int J Rehabil Res* 2015; **38**(1): 49-54.

245. Langeskov-Christensen M, Hvid LG, Jensen HB, et al. Efficacy of high-intensity aerobic exercise on common multiple sclerosis symptoms. *Acta Neurol Scand* 2021.

246. Torres-Costoso A, Martinez-Vizcaino V, Reina-Gutierrez S, et al. Effect of Exercise on Fatigue in Multiple Sclerosis: A Network Meta-analysis Comparing Different Types of Exercise. *Arch Phys Med Rehabil* 2021.

247. Raats J, Lamers I, Merken I, et al. The content and effects of trunk rehabilitation on trunk and upper limb performance in people with Multiple Sclerosis: a systematic review. *Eur J Phys Rehabil Med* 2021.

248. Reina-Gutierrez S, Cavero-Redondo I, Martinez-Vizcaino V, et al. The type of exercise most beneficial for quality of life in people with multiple sclerosis: A network meta-analysis. *Ann Phys Rehabil Med* 2021; **65**(3): 101578.

249. Calabro RS, Cassio A, Mazzoli D, et al. What does evidence tell us about the use of gait robotic devices in patients with multiple sclerosis? A comprehensive systematic review on functional outcomes and clinical recommendations. *Eur J Phys Rehabil Med* 2021; **57**(5): 841-9.

250. Wuerch E, Lozinski B, Yong VW. MedXercise: a promising strategy to promote remyelination. *Curr Opin Pharmacol* 2021; **61**: 120-6.

251. Cree BAC, Oksenberg JR, Hauser SL. Multiple sclerosis: two decades of progress. *Lancet Neurol* 2022; **21**(3): 211-4.

252. Absinta M, Sati P, Masuzzo F, et al. Association of Chronic Active Multiple Sclerosis Lesions With Disability In Vivo. *JAMA Neurol* 2019; **76**(12): 1474-83.

253. Minagar A, Barnett MH, Benedict RH, et al. The thalamus and multiple sclerosis: modern views on pathologic, imaging, and clinical aspects. *Neurology* 2013; **80**(2): 210-9.

254. Burggraaff J, Liu Y, Prieto JC, et al. Manual and automated tissue segmentation confirm the impact of thalamus atrophy on cognition in multiple sclerosis: A multicenter study. *Neuroimage Clin* 2021; **29**: 102549.

255. Ontaneda D, Raza PC, Mahajan KR, et al. Deep grey matter injury in multiple sclerosis: a NAIMS consensus statement. *Brain* 2021; **144**(7): 1974-84.

256. Haider L, Simeonidou C, Steinberger G, et al. Multiple sclerosis deep grey matter: the relation between demyelination, neurodegeneration, inflammation and iron. *J Neurol Neurosurg Psychiatry* 2014; **85**(12): 1386-95.

257. Pontillo G, Cocozza S, Lanzillo R, et al. Determinants of Deep Gray Matter Atrophy in Multiple Sclerosis: A Multimodal MRI Study. *AJNR Am J Neuroradiol* 2019; **40**(1): 99-106.

258. Taege Y, Hagemeier J, Bergsland N, et al. Assessment of mesoscopic properties of deep gray matter iron through a model-based simultaneous analysis of magnetic susceptibility and R2* - A pilot study in patients with multiple sclerosis and normal controls. *Neuroimage* 2019; **186**: 308-20.

259. Elkady AM, Cobzas D, Sun H, Seres P, Blevins G, Wilman AH. Five year iron changes in relapsing-remitting multiple sclerosis deep gray matter compared to healthy controls. *Mult Scler Relat Disord* 2019; **33**: 107-15.

260. Pontillo G, Petracca M, Monti S, et al. Unraveling Deep Gray Matter Atrophy and Iron and Myelin Changes in Multiple Sclerosis. *AJNR Am J Neuroradiol* 2021; **42**(7): 1223-30.

261. Mills EA, Begay JA, Fisher C, Mao-Draayer Y. Impact of trial design and patient heterogeneity on the identification of clinically effective therapies for progressive MS. *Mult Scler* 2018; **24**(14): 1795-807.

262. Pontillo G, Petracca M, Cocozza S, Brunetti A. The Development of Subcortical Gray Matter Atrophy in Multiple Sclerosis: One Size Does Not Fit All. *AJNR Am J Neuroradiol* 2020; **41**(9): E80-E1.

263. Yong HYF, Yong VW. Mechanism-based criteria to improve therapeutic outcomes in progressive multiple sclerosis. *Nat Rev Neurol* 2022; **18**(1): 40-55.

264. Kerbrat A, Gros C, Badji A, et al. Multiple sclerosis lesions in motor tracts from brain to cervical cord: spatial distribution and correlation with disability. *Brain* 2020; **143**(7): 2089-105.

265. Altermatt A, Gaetano L, Magon S, et al. Clinical Correlations of Brain Lesion Location in Multiple Sclerosis: Voxel-Based Analysis of a Large Clinical Trial Dataset. *Brain Topogr* 2018; **31**(5): 886-94.

266. Sorensen PS, Sellebjerg F, Hartung HP, Montalban X, Comi G, Tintore M. The apparently milder course of multiple sclerosis: changes in the diagnostic criteria, therapy and natural history. *Brain* 2020; **143**(9): 2637-52.

267. Barzegar M, Najdaghi S, Afshari-Safavi A, Nehzat N, Mirmosayyeb O, Shaygannejad V. Early predictors of conversion to secondary progressive multiple sclerosis. *Mult Scler Relat Disord* 2021; **54**: 103115.

268. Healy BC, Zurawski J, Chitnis T, Weiner HL, Glanz BI. Patient-reported outcomes associated with transition to secondary progressive multiple sclerosis. *Qual Life Res* 2021.

269. Iaffaldano P, Lucisano G, Patti F, et al. Transition to secondary progression in relapsing-onset multiple sclerosis: Definitions and risk factors. *Mult Scler* 2021; **27**(3): 430-8.

270. Dwyer MG, Bergsland N, Ramasamy DP, Jakimovski D, Weinstock-Guttman B, Zivadinov R. Atrophied Brain Lesion Volume: A New Imaging Biomarker in Multiple Sclerosis. *J Neuroimaging* 2018; **28**(5): 490-5.

271. Zivadinov R, Horakova D, Bergsland N, et al. A Serial 10-Year Follow-Up Study of Atrophied Brain Lesion Volume and Disability Progression in Patients with Relapsing-Remitting MS. *AJNR Am J Neuroradiol* 2019; **40**(3): 446-52.

272. Genovese AV, Hagemeier J, Bergsland N, et al. Atrophied Brain T2 Lesion Volume at MRI Is Associated with Disability Progression and Conversion to Secondary Progressive Multiple Sclerosis. *Radiology* 2019; **293**(2): 424-33.

273. Zivadinov R, Bergsland N, Dwyer MG. Atrophied brain lesion volume, a magnetic resonance imaging biomarker for monitoring neurodegenerative changes in multiple sclerosis. *Quant Imaging Med Surg* 2018; **8**(10): 979-83.

274. Bergsland N, Dwyer MG, Jakimovski D, Weinstock-Guttman B, Zivadinov R. Diffusion tensor imaging reveals greater microstructure damage in lesional tissue that shrinks into cerebrospinal fluid in multiple sclerosis. *J Neuroimaging* 2021; **31**(5): 995-1002.

275. Paul F, Calabresi PA, Barkhof F, et al. Optical coherence tomography in multiple sclerosis: A 3-year prospective multicenter study. *Ann Clin Transl Neurol* 2021; **8**(12): 2235-51.

276. Petzold A, Balcer LJ, Calabresi PA, et al. Retinal layer segmentation in multiple sclerosis: a systematic review and meta-analysis. *Lancet Neurol* 2017; **16**(10): 797-812.

277. Zivadinov R, Tavazzi E, Hagemeier J, et al. The Effect of Glatiramer Acetate on Retinal Nerve Fiber Layer Thickness in Patients with Relapsing-Remitting Multiple Sclerosis: A Longitudinal Optical Coherence Tomography Study. *CNS Drugs* 2018; **32**(8): 763-70.

278. El Ayoubi NK, Ghassan S, Said M, Allam J, Darwish H, Khoury SJ. Retinal measures correlate with cognitive and physical disability in early multiple sclerosis. *J Neurol* 2016; **263**(11): 2287-95.

279. Birkeldh U, Manouchehrinia A, Hietala MA, et al. Retinal nerve fiber layer thickness associates with cognitive impairment and physical disability in multiple sclerosis. *Mult Scler Relat Disord* 2019; **36**: 101414.

280. Bsteh G, Berek K, Hegen H, et al. Serum neurofilament levels correlate with retinal nerve fiber layer thinning in multiple sclerosis. *Mult Scler* 2020; **26**(13): 1682-90.

281. Sepulcre J, Murie-Fernandez M, Salinas-Alaman A, Garcia-Layana A, Bejarano B, Villoslada P. Diagnostic accuracy of retinal abnormalities in predicting disease activity in MS. *Neurology* 2007; **68**(18): 1488-94.

282. Fisher JB, Jacobs DA, Markowitz CE, et al. Relation of visual function to retinal nerve fiber layer thickness in multiple sclerosis. *Ophthalmology* 2006; **113**(2): 324-32.

283. Dogan U, Ulas F, Turkoglu SA, Ogun MN, Agca S. Eyes are mirror of the brain: comparison of multiple sclerosis patients and healthy controls using OCT. *Int J Neurosci* 2019; **129**(9): 848-55.

284. Gabilondo I, Martinez-Lapiscina EH, Martinez-Heras E, et al. Trans-synaptic axonal degeneration in the visual pathway in multiple sclerosis. *Ann Neurol* 2014; **75**(1): 98-107.

285. Uzunkopru C, Yuceyar N, Yilmaz SG, Afrashi F, Ekmekci O, Taskiran D. Retinal Nerve Fiber Layer Thickness Correlates with Serum and Cerebrospinal Fluid Neurofilament Levels and is Associated with Current Disability in Multiple Sclerosis. *Noro Psikiyatr Ars* 2021; **58**(1): 34-40.

286. Guerrieri S, Comi G, Leocani L. Optical Coherence Tomography and Visual Evoked Potentials as Prognostic and Monitoring Tools in Progressive Multiple Sclerosis. *Front Neurosci* 2021; **15**: 692599.

287. Fujimori J, Nakashima I. Serum neurofilament light is a sensitive biomarker that reflects grey matter volume in Japanese patients with multiple sclerosis. *J Neurol Sci* 2021; **427**: 117528.

288. Jakimovski D, Zivadinov R, Vaughn CB, Ozel O, Weinstock-Guttman B. Clinical effects associated with five-year retinal nerve fiber layer thinning in multiple sclerosis. *J Neurol Sci* 2021; **427**: 117552.

289. Garzone D, Finger RP, Mauschitz MM, Santos MLS, Breteler MMB, Aziz NA. Neurofilament light chain and retinal layers' determinants and association: A population-based study. *Ann Clin Transl Neurol* 2022.

290. Jakimovski D, Benedict RHB, Weinstock-Guttman B, et al. Visual deficits and cognitive assessment of multiple sclerosis: confounder, correlate, or both? *J Neurol* 2021; **268**(7): 2578-88.

291. Bonzano L, Pedulla L, Tacchino A, et al. Upper limb motor training based on task-oriented exercises induces functional brain reorganization in patients with multiple sclerosis. *Neuroscience* 2019; **410**: 150-9.

292. Peran P, Nemmi F, Dutilleul C, et al. Neuroplasticity and brain reorganization associated with positive outcomes of multidisciplinary rehabilitation in progressive multiple sclerosis: A fMRI study. *Mult Scler Relat Disord* 2020; **42**: 102127.

293. Boffa G, Tacchino A, Sbragia E, et al. Preserved brain functional plasticity after upper limb task-oriented rehabilitation in progressive multiple sclerosis. *Eur J Neurol* 2020; **27**(1): 77-84.

294. Akbar N, Sandroff BM, Wylie GR, et al. Progressive resistance exercise training and changes in resting-state functional connectivity of the caudate in persons with multiple sclerosis and severe fatigue: A proof-of-concept study. *Neuropsychol Rehabil* 2020; **30**(1): 54-66.

295. Guerrera S, Morabito R, Baglieri A, et al. Cortical reorganization in multiple sclerosis after intrathecal baclofen therapy. *Neurocase* 2014; **20**(2): 225-9.

296. Nava E, Roder B. Adaptation and maladaptation insights from brain plasticity. *Prog Brain Res* 2011; **191**: 177-94.

297. Trojan S, Pokorny J. Theoretical aspects of neuroplasticity. *Physiol Res* 1999; **48**(2): 87-97.

298. De Giglio L, Tona F, De Luca F, et al. Multiple Sclerosis: Changes in Thalamic Resting-State Functional Connectivity Induced by a Home-based Cognitive Rehabilitation Program. *Radiology* 2016; **280**(1): 202-11.

299. Negaresh R, Motl RW, Zimmer P, Mokhtarzade M, Baker JS. Effects of exercise training on multiple sclerosis biomarkers of central nervous system and disease status: a systematic review of intervention studies. *Eur J Neurol* 2019; **26**(5): 711-21.

300. Prosperini L, Di Filippo M. Beyond clinical changes: Rehabilitation-induced neuroplasticity in MS. *Mult Scler* 2019; **25**(10): 1348-62.

301. Rocca MA, Preziosa P, Filippi M. Application of advanced MRI techniques to monitor pharmacologic and rehabilitative treatment in multiple sclerosis: current status and future perspectives. *Expert Rev Neurother* 2019; **19**(9): 835-66.

302. Dalgas U, Langeskov-Christensen M, Stenager E, Riemenschneider M, Hvid LG. Exercise as Medicine in Multiple Sclerosis-Time for a Paradigm Shift: Preventive, Symptomatic, and Disease-Modifying Aspects and Perspectives. *Curr Neurol Neurosci Rep* 2019; **19**(11): 88.

303. Centonze D, Leocani L, Feys P. Advances in physical rehabilitation of multiple sclerosis. *Curr Opin Neurol* 2020; **33**(3): 255-61.

304. Sandroff BM, Jones CD, Baird JF, Motl RW. Systematic Review on Exercise Training as a Neuroplasticity-Inducing Behavior in Multiple Sclerosis. *Neurorehabil Neural Repair* 2020; **34**(7): 575-88.

305. Mark VW. Retention of Physical Gains in the Community Following Physical Training for Multiple Sclerosis: A Systematic Review and Implications. *Semin Neurol* 2021; **41**(2): 177-88.

306. Marek S, Tervo-Clemmens B, Calabro FJ, et al. Reproducible brain-wide association studies require thousands of individuals. *Nature* 2022; **603**(7902): 654-60.

307. Jain P, Chaney AM, Carlson ML, Jackson IM, Rao A, James ML. Neuroinflammation PET Imaging: Current Opinion and Future Directions. *J Nucl Med* 2020; **61**(8): 1107-12.

Appendix 1

MRI biomarkers of disease progression and conversion to secondary progressive multiple sclerosis

Abstract

Introduction: Conventional imaging measures remain a key clinical tool for the diagnosis multiple sclerosis (MS) and monitoring of patients. However, most measures used in the clinic show unsatisfactory performance in predicting disease progression and conversion to secondary progressive MS.

Areas covered: Sophisticated imaging techniques have facilitated the identification of imaging biomarkers associated with disease progression, such as global and regional brain volume measures, and with conversion to secondary progressive MS, such as leptomeningeal contrast enhancement and chronic inflammation. The relevance of emerging imaging approaches partially overcoming intrinsic limitations of traditional techniques is also discussed.

Expert opinion: Imaging biomarkers capable of detecting tissue damage early on in the disease, with the potential to be applied in multicenter trials and at an individual level in clinical settings, are strongly needed. Several measures have been proposed, which exploit advanced imaging acquisitions and/or incorporate sophisticated post-processing, can quantify irreversible tissue damage. The progressively wider use of high-strength field MRI and the development of more advanced imaging techniques will help capture the missing pieces of the MS puzzle. The ability to more reliably identify those at risk for disability progression will allow for earlier intervention with the aim to favorably alter the disease course.

Appendix 2

Targeting Iron Dyshomeostasis for Treatment of Neurodegenerative Disorders

Abstract

While iron has an important role in the normal functioning of the brain owing to its involvement in several physiological processes, dyshomeostasis has been found in many neurodegenerative disorders, as evidenced by both histopathological and imaging studies. Although the exact causes have remained elusive, the fact that altered iron levels have been found in disparate diseases suggests that iron may contribute to their development and/or progression. As such, the processes involved in iron dyshomeostasis may represent novel therapeutic targets. There are, however, many questions about the exact interplay between neurodegeneration and altered iron homeostasis. Some insight can be gained by considering the parallels with respect to what occurs in healthy aging, which is also characterized by increased iron throughout many regions in the brain along with progressive neurodegeneration. Nevertheless, the exact mechanisms of iron-mediated damage are likely disease specific to a certain degree, given that iron plays a crucial role in many disparate biological processes, which are not always affected in the same way across different neurodegenerative disorders. Moreover, it is not even entirely clear yet whether iron actually has a causative role in all of the diseases where altered iron levels have been noted. For example, there is strong evidence of iron dyshomeostasis leading to neurodegeneration in Parkinson's disease, but there is still some question as to whether changes in iron levels are merely an epiphenomenon in multiple sclerosis. Recent advances in neuroimaging now offer the possibility to detect and monitor iron levels in vivo, which allows for an improved understanding of both the temporal and spatial dynamics of iron changes and associated neurodegeneration compared to post-mortem studies. In this regard, iron-based imaging will likely play an important role in the development of therapeutic approaches aimed at addressing altered iron dynamics in neurodegenerative diseases. Currently, the bulk of such therapies have focused on chelating excess iron. Although there is some evidence that these treatment options may yield some benefit, they are not without their own limitations. They are generally effective at reducing brain iron levels, as assessed by imaging, but clinical benefits are more modest. New drugs that specifically target iron-related pathological processes may offer the possibility to prevent, or at the least, slow down irreversible neurodegeneration, which represents an unmet therapeutic target.