UNIVERSITÀ DEGLI STUDI DI GENOVA

SCUOLA DI SCIENZE MEDICHE E FARMACEUTICHE

Tesi di Laurea in Medicina e Chirurgia

The impact of autologous bone marrow transplantation on brain microstructure in aggressive multiple sclerosis assessed by quantitative MRI

Dipartimento di Neuroscienze, Riabilitazione, Oftalmologia, Genetica e Scienze Materno-Infantili (DINOGMI)

Relatrice:
Prof.ssa Matilde Inglese

Candidato:
Jacopo Celada Ballanti

Correlatore:
Dott. Giacomo Boffa

Anno accademico 2022/2023

A Giovanni,
"Non aspirare alla vita immortale,
ma esaurisci il campo del possibile".

Pindaro

INDEX

DISCUSSION...43

REFERENCES 46

RINGRAZIAMENTI 56

ABSTRACT

AIM: Autologous hematopoietic stem cell transplant (AHSCT) has proven to be an effective treatment for aggressive multiple sclerosis (MS). It is associated with significant risks and an increased rate of brain atrophy in the first months after the procedure has been observed using MRI. Whether this phenomenon is caused by drug-induced neurotoxicity or pseudoatrophy is unclear. This study aimed to gain a better understanding of the impact of AHSCT on brain microstructure by analyzing MRI data in MS patients treated with AHSCT.

METHODS: MRI data were collected from 17 patients with aggressive MS who underwent AHSCT between 2006 and 2019 at 2 Italian MS Centers. Patients received an intermediate-intensity conditioning regimen (16 BEAM+ATG, 1 CY+ATG). MRI protocol included high-resolution fast-spoiled-gradient-echo 3D T1-weighted sequence, 3D FLAIR, and axial single-shot spin-echo echo-planar diffusion tensor imaging (DTI). Brain volumes (total brain -TB-, gray matter -GM-, and white matter -WM-) were derived using CAT-12, part of SPM. Fractional anisotropy (FA) values were obtained from DTI.

RESULTS: Volumetric analyses included 14 subjects with 3 MRI scans (mean follow-up: 626 days) and 8 subjects with 6 MRI scans (mean follow-up: 1794 days). A significant TBV loss was detected in the first 6-12 months after AHSCT, with decreased WM volumes ($p=0.003$). At the same timepoints, FA, which reflects WM integrity, remained unaltered. After the initial accelerated TBV loss, brain atrophy slowed down and reached levels comparable to normal aging.

CONCLUSION: Significant early TBV loss is common after AHSCT and is mainly driven by a reduction in WM volume. In these regions, diffusivity maps obtained from DTI showed no microstructural changes, suggesting that neurotoxicity from chemotherapy is not the main driver of BVL in these patients. After an initial accelerated brain atrophy, volume loss slows down to levels comparable to normal aging.

Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory-degenerative autoimmune disease that primitively affects the central nervous system causing progressive demyelination and subsequent neuronal suffering

Within the family of demyelinating disorders, it represents the most common entity and is currently the leading cause of neurological disability in young and middle-aged people. Most recent estimates indicate a worldwide prevalence of 2,8 million people affected[1], of which there are currently 133,000 cases in Italy with an incidence of about 3,600 new diagnoses each year[2]. Previously it was believed that the incidence of Multiple Sclerosis was higher in countries with high latitudes, but more recent studies have discarded this hypothesis[3]. In line with this, Italy today is considered a high-risk country for developing MS[4]. It is important to note that many aspects contribute to the wide variation of the prevalence of this disease in different countries, of which the main ones recognized are the genetics of the population, the subject's interaction with the environment, and the presence of medical facilities capable of establishing a diagnosis, although survival is an essential factor that can be taken into consideration for developed countries.

The disease's etiology is unknown; however, subject-related risk factors such as sex, age, and genetic predisposition play a significant role and are related to environmental interaction. It is known that the disease tends to appear more frequently in female individuals with an F:M ratio ranging from 2:1 to 3:1[5]. This phenomenon, although also found in other autoimmune diseases, still has no explanation, but it is believed to be due to the influence of sex-linked genetic factors and hormonal factors. The disease strikes, on average, around the age of 30 years[6]. The strongest genetic influence in MS is mediated by the HLA class II genes, especially HLA–DR2b; regarding other genetic factors, the presence of the HLA-DBR1*15:01 haplotype has been recognized as a predisposing condition: individuals carrying this variant are three times more likely to develop the disease.

Several environmental factors are associated with the development of MS, the most important ones being obesity, vitamin D deficiency, cigarette smoking, and Epstein-Barr virus (EBV) infection, especially in adulthood[7].

Multiple Sclerosis's pathogenesis derives from the immune system's uncontrolled activation against antigens expressed exclusively in the central nervous system. The immune response is directed against myelin components (especially MBP, myelin basic protein) and other neuronal elements. Some hypotheses suggest that a mechanism of molecular mimicry with antigens of viral origin may be the underlying cause of the genesis of the inflammatory process[8]. Adaptive immunity is thought to play a crucial role in activating and maintaining the inflammatory process: CD4+ T lymphocytes, once activated by antigen presentation, invade the CNS, trespassing the blood-brain barrier and triggering an immune cascade involving CD8+ T cells and NK cells. Mainly Type 1 T helper cells (Th1) are involved in the immune response, but studies show the involvement of the Th17 cells[9]. Although it was once believed that B lymphocytes were not relevant in pathogenesis, it is now known that they contribute to the pathogenetic process by acting as antigen-presenting cells (APCs) along with macrophages and dendritic cells.

Immunologic activation results in the appearance of focal white matter lesions (WML), the prominent pathogenetic expression of the disease. These lesions typically occur in the periventricular white matter, brainstem, optic nerve, and spinal cord (Figure 1). Demyelination also affects gray matter, characterizing cortical and subcortical lesions (GML) that are now known to occur not only in advanced stages but already in the early stages of the disease[10].
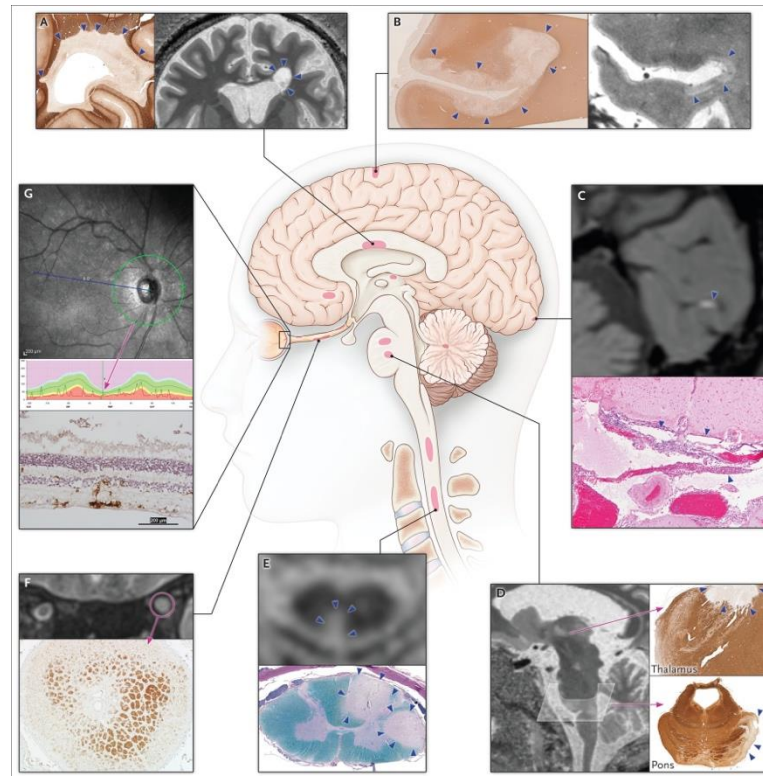
Figure 1: Topography of Multiple Sclerosis Lesions. Shown is a schematic of lesion location, calling out imaging and pathological examples in the periventricular white matter (inset A), subpial cortex (B), leptomeninges (C), thalamus and pons (D), spinal cord (E), optic nerve (F), and retina (G)[11]. Reproduced with permission from *Multiple Sclerosis. Daniel S. Reich, M.D., Ph.D., Claudia F. Lucchinetti, M.D., and Peter A. Calabresi, M.D.* Copyright Massachusetts Medical Society. Figure 2-3: Shown are some

From a clinical perspective, Multiple Sclerosis can take on heterogeneous features in clinical presentation and progression over time.

The first manifestation may be sudden or insidious. If recognized clinically, it is classified as *clinically isolated syndrome* (CIS), a form associated with a high risk of developing the disease, or *radiologically isolated syndrome* (RIS) if evidenced incidentally on imaging examinations. *Relapsing-remitting Multiple Sclerosis* (RRMS) is the most common and occurs in 85-90% of cases, in which case there is an acute neurological manifestation with complete remission of symptoms between episodes. In 15-30% of cases, the relapsing-remitting form tends to evolve within 20-25 years into a *secondary progressive Multiple Sclerosis* (SPMS), where permanent neurological deficits accumulate between disease flare-ups[12]. In 10% of cases, the disease may manifest as *primary progressive Multiple Sclerosis* (PPMS), with symptoms gradually worsening from the onset without relapses or remissions. The

current consensus[13] characterizes progressive MS based on disease progression and activity, defining the phenotypes that are shown in Figure 2.
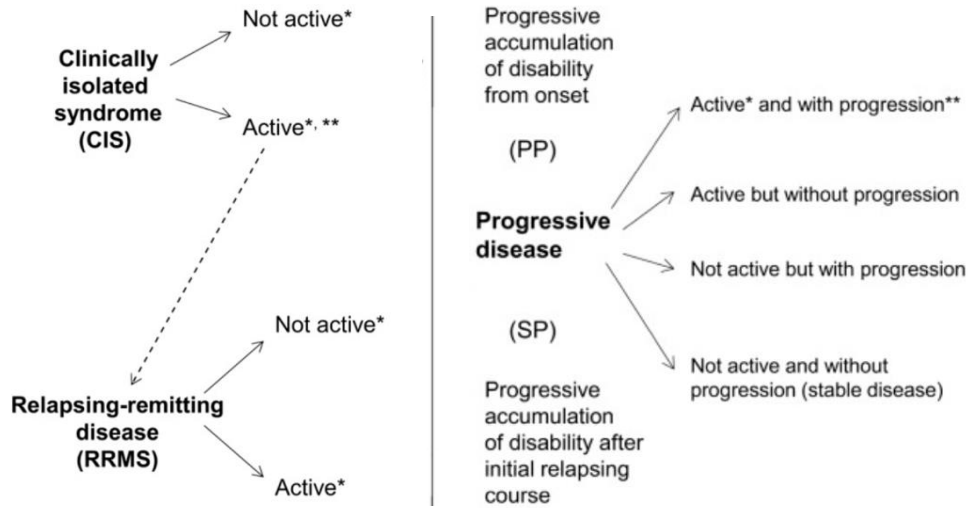

Figure 2: The 2013 multiple sclerosis phenotype descriptions for relapsing and progressive disease[13].

Typical disease onset includes optic neuritis, double vision, facial sensory loss, cerebellar ataxia and nystagmus, partial myelopathy, paresthesia or anesthesia, asymmetric limb weakness, urge incontinence, and Lhermitte sign (an electric shock-like sensation that radiates down the back into the legs typically induced by flexion or other movements of the neck)[6]. Despite this, multiple sclerosis may begin by manifesting atypically with other neurologic symptoms. Because cortical and subcortical gray matter lesions can occur early, cognitive symptoms, once considered more typical of advanced forms, can also appear in the initial stages[14].

Symptoms	Percentage of cases	Symptoms	Percentage of cases
Loss of sensibility	37	Lhermitte sign	3
Optic neuritis	36	Pain	3
Weakness	35	Dementia	2
Paresthesia	24	Vision loss	2
Diplopia	15	Facial paralysis	1
Ataxia	11	Impotence	1
Vertigo	6	Myokymia	1
Paroxysmal symptoms	4	Epilepsy	1
Bladder symptoms	4	Falls	1

Table 1: Initial symptoms of MS[15].

The diagnosis of Multiple Sclerosis derives from the integration of clinical, laboratory and imaging data. To reach diagnostic confirmation, it is necessary to demonstrate the presence of two fundamental factors: dissemination in space and dissemination in time (development of new lesions spaced out over time). Dissemination in space can be demonstrated by the presence of 2 or more lesions at different sites in brain MRI, whereas dissemination over time, the development of new lesions spaced out over time, required at least two acute clinical episodes before the use of gadolinium to highlight active lesions. Gadolinium contrast agent allows the identification of active lesions in loci in the central nervous system where acute inflammation generates a disruption of the blood-brain barrier[16]. The simultaneous presence of an active lesion and a lesion that does not impregnate with gadolinium confirms dissemination over time in McDonald and MAGNIMS criteria, allowing the establishment of a diagnosis even in a typical CIS[17]. The use of clinical criteria and documentation of oligoclonal bands on CSF examination are supportive elements that contribute to the diagnosis.

DIT and DIS in CIS: Clinical Scenarios	Further Clinical, MRI or CSF criteria required to meet DIT and DIS
Scenario 1: DIT met, but DIS NOT met Two or more attacks (DIT met); and clinical evidence of 1 lesion (DIS not met)	DIS should be shown by **1** of the following criteria: • Additional clinical attack involving lesion in different CNS site • MRI evidence of ≥1 T2 lesion(s) in ≥2 of the 4 typical CNS sites (periventricular, cortical/juxtacortical, infratentorial and spinal cord)
Scenario 2: DIT NOT met; DIS met One attack (DIT not met); and clinical evidence of ≥2 lesions (DIS met)	DIT should be shown by **1** of the following criteria: • Additional clinical attack • MRI showing simultaneous gadolinium enhancing and nonenhancing typical MS lesions; or • Follow up MRI showing new T2 lesion or gadolinium enhancing typical MRI lesion in comparison with baseline scan (timing of baseline scan is disregarded) • CSF oligoclonal bands
Scenario 3: DIT and DIS both NOT met One attack (DIT not met); and clinical evidence of 1 lesion (DIS not met)	• DIT should be shown by **1** of the criteria in *scenario 2* • DIS should be shown by **1** of the criteria in *scenario 1*
Scenario 4: DIT and DIS both met **A]** Two or more attacks (DIT met); and clinical evidence of ≥2 lesions (DIS met) **B]** Two or more attacks (DIT met); and clinical evidence of 1 lesion along with clear-cut historical evidence of prior attack involving lesion in different CNS site (DIS met)	No further criteria needed

CIS, clinically isolated syndrome; DIT, dissemination in time; DIS, dissemination in space.

Table 2A: 2017 McDonald criteria for MS diagnosis. The criteria require demonstration of the CNS lesions being disseminated in time and space; and other more likely alternative diagnoses should be eliminated[18].

Dissemination in Space (DIS)	Revised MAGNIMS consensus guidelines incorporated or not in 2017 McDonald criteria	Any change in 2017 McDonald criteria compared to 2010 McDonald criteria in concordance with 2016 MAGNIMS consensus guidelines
DIS can be demonstrated by the involvement of at least 2 out of 5 areas of the CNS as follows:	No	No change from 4 areas in 2010 McDonald criteria- Newly proposed Optic nerve was not incorporated in 2017 McDonald criteria
1) ≥3 periventricular lesions	No	No change from1 periventricular lesion of 2010 McDonald criteria
2) ≥1 infratentorial lesion (Symptomatic or asymptomatic)	Yes	No change in number of lesion(s) in 2010 McDonald criteria, but symptomatic lesions excluded in 2010 are now included in 2017 McDonald criteria
3) ≥1 spinal cord lesion (Symptomatic or asymptomatic)	Yes	No change in number of lesion(s) in 2010 McDonald criteria, but symptomatic lesions excluded in 2010 are now included in 2017 McDonald criteria
4) ≥1 optic nerve lesion	No	No change from 2010 McDonald criteria
5) ≥1 cortical/juxtacortical lesion (involvement of white matter next to cortex and/or cortex, thereby expanding the term "juxtacortical" lesion)	Yes	Cortical lesion was added in addition to the juxtacortical lesion of 2010 McDonald criteria as a combined 'cortical/juxtacortical' lesion in 2017 McDonald criteria
Dissemination in Time (DIT)	*Revised MAGNIMS consensus guidelines incorporated or not in 2017 McDonald criteria*	*Any change in 2017 McDonald criteria compared to 2010 McDonald criteria in concordance with 2016 MAGNIMS consensus guidelines*
Both symptomatic and asymptomatic lesions can be used to demonstrate (DIS or) DIT	Yes	Symptomatic lesions excluded in 2010 are now included in 2017 McDonald criteria

Table 2B: Revised 2016 MAGNIMS consensus guidelines compared to revised 2017 McDonald criteria and the older 2010 McDonald criteria. Revised 2016 MAGNIMS consensus guidelines and its incorporation in 2017 McDonald criteria[18].

Once the diagnosis has been established, patients should be followed up over time to check for the absence of new clinical relapses, confirmed disability progression (CDP, defined as an increase in EDSS score of 1. 5 points from a baseline score of 0, of 1.0 point from a baseline score of 1.0 or more or of 0.5 points from a baseline score of greater than 5.0[19]), new T2W or Gd+ lesions, and the development of brain atrophy, defined as mean AR-BVL of more than 0.4%[20]. These objectives are defined as NEDA, "No Evidence of Disease Activity".

1.2 Aggressive Multiple Sclerosis

Aggressive Multiple Sclerosis is a disease subgroup characterized by more frequent and violent clinical relapses, an increased rate of disability, and a decreased response to conventional therapies[21]. Although no commonly accepted definition of aggressive MS exists, multiple attempts have been made, and several criteria have been used.

Despite the absence of well-defined criteria, it is estimated that the proportion of patients with aggressive MS is 4-14%[22], meaning that between 5.200-18.600 people in Italy present with this form. The first attempt to characterize this group of patients was "malignant multiple sclerosis", which was defined as "disease with a rapid progressive course, leading to significant disability in multiple neurologic systems or death in a relatively short time after disease onset"[23]. This definition was too unspecific and did not contribute to a correct evaluation of the patients, so other authors classified "ever

malignant multiple sclerosis" patients who reached an Expanded-Disability-Status-Scale (EDS) score of 6 within 5 years from disease onset[24]. These patients had significantly more relapses, more motor symptoms, and a higher frequency of progressive onset. Other authors[25] describe aggressive MS as: (a) patients who reached an EDSS of 6 within 5 years from disease onset; or (b) patients who reached an EDSS score of 6 before they are 40 years old; or (c) patients converted to a secondary progressive MS phenotype within 3 years from disease onset.

However, the proposed definitions limited the ability to make early recognition of the condition and the application of rapid and effective treatment choices because they required a retrospective assessment of the disease course or a prolonged prospective evaluation until the fulfillment of the criteria.

More recent definitions described aggressive MS as relapsing MS with one or more of the following features: (a) EDSS score of 4.0 within 5 years of onset; (b) Multiple ($\geqslant$2) relapses with incomplete resolution in the past year; (c) $\geqslant$2 MRI scans showing new or enlarging T2 lesions or Gd+ lesions despite treatment; (d) No response to therapy with one or more DMTs for up to 1 year[26]. Other definitions of aggressive MS have been proposed when considering treatment options. Saccardi et al.[27], aiming at identifying patients eligible for hematopoietic stem cell transplantation, defined "highly active MS" as "failure of at least one and up to three active DMT evidenced by ongoing or increased clinical and MRI activity". A study in CIS patients showed that a percentage brain volume change (PBVC) decrease below -0.817% in the first year of disease evolution was an independent predictor of a shorter time to a second attack[28].

Although there is still no universal definition of aggressive multiple sclerosis, several studies have identified poor prognostic factors that can lead to the development of more aggressive forms of the disease, facilitating early recognition and diagnosis.

- **Clinical features**
  - *Demographics*: Male sex; Older age (>40 years) at onset; African American; African–Latin American.
  - *Relapse severity*: ≥1-point change on EDSS, ≥2-point change on any individual functional system, or ≥1-point change on any two functional systems; Steroid requirement; Hospitalization.
  - *Type of attack*: Multifocal; Partial or incomplete recovery; Attack affects motor, cerebellar, sphincteric or cognitive functions.
  - *Relapse frequency*: Frequent relapses in the first 2–5 years; Short interattack interval.
  - *Disease course*: Rapid accrual of disability, e.g. EDSS score of 3.0 within 5 years, with superimposed relapses.
- **MRI features**
  - *At onset*: High T2 lesion burden; More than two gadolinium-enhancing lesions; Presence of T1 lesions ('black holes'); Early discernable atrophy; Infratentorial lesions
  - *At follow-up*: Presence of new T2 lesions; One or more new gadolinium-enhancing lesions

Factors that might suggest aggressive MS[26]

In addition to the problematic recognition of patients with aggressive MS, to date, there are still no evidence-based criteria for the proper management of these patients. Furthermore, the treatment of this condition is neither addressed in the guidelines of the American Academy of Neurology[29], nor the European Academy of Neurology/European Committee on Treatment and Research in MS[30]. Patients with aggressive MS are underrepresented in typical randomized clinical trials, due to the strict inclusion/exclusion criteria of sponsored clinical trials and the ethical dilemma of blind randomization of a patient with such an aggressive disease[31].

The lack of data on aggressive MS raises severe problems regarding treatment and prognosis for this subpopulation: more information is needed regarding the efficacy of immunosuppressive therapies and long-term outcomes.
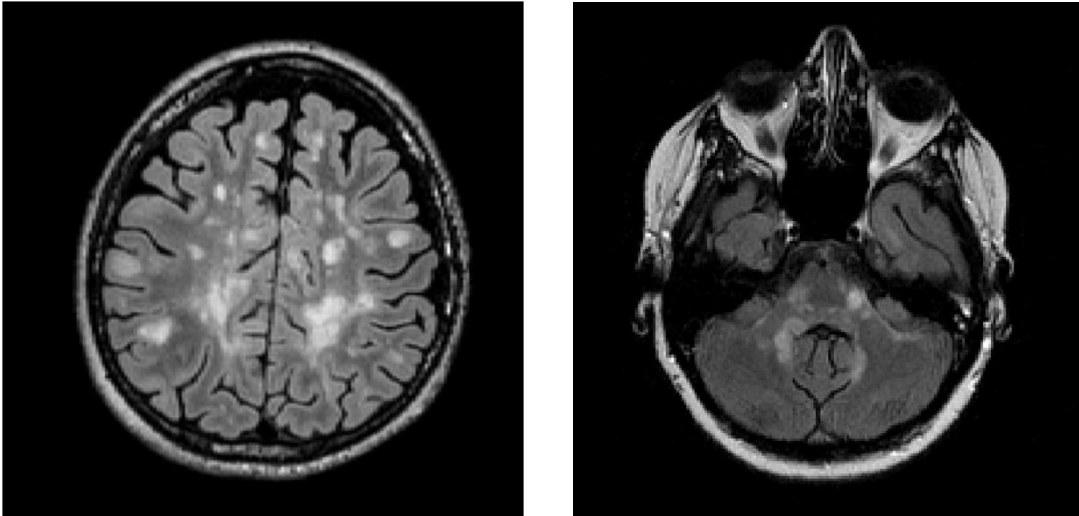
Figure 2-3: Shown are some examples of MRI scans of two patients with aggressive MS. These patients often present with a high lesion load, expression of the severity of the disease. Figure 3 on the right shows an extensive infratentorial involvement with multiple brainstem lesions, often associated with disability.

1.3 High efficacy treatment in Multiple Sclerosis

Treatment of multiple sclerosis is based on shutting down the immune system through immunosuppressive drugs. Treatment goals are to extinguish clinical relapses, treat specific symptoms, and maintain a state of remission. Thus, a distinction is made between acute phase treatment and chronic treatment, which is based on medications called "disease-modifying drugs".

Relapse Treatments

The aim of relapse treatment is to accelerate clinical recovery, as no effect on the long-term prognosis of multiple sclerosis is expected[1]. Symptomatic treatments are aimed at maintaining function and improving quality of life. It is common practice to treat acute relapses of MS with a short course (typically 3 to 5 days) of a corticosteroid that has a rapid onset of action and produces few adverse drug effects, such as intravenous (IV) methylprednisolone or dexamethasone[33]. In cases of steroid-resistant multiple sclerosis relapses, after a second course of high-dose intravenous methylprednisolone, the most common intervention is plasma exchange (PLEX)[34] which leads to a positive response in 72% of patients[35].

Disease-modifying Therapies

Disease-modifying therapies (DMT) are used to keep the disease in a quiescent state, prevent the development of new relapses and slow down the evolution of MS. To date, there are many drugs belonging to this category, and their number is constantly increasing. Dividing them according to function, it can be possible to identify drugs with predominantly immunosuppressive function (such as cladribine, alemtuzumab and ocrelizumab) or with immunomodulatory function (such as interferon beta and glatiramer acetate)[36]. A table of DMTs approved by the FDA and EMA is shown below (Table 3).

Drug name	Brand name	Mechanism of action	Route of administration	Approved US indication	US approval year	EU approval year
Infusion/monoclonal therapies						
Ofatumumab	Kesimpta®	Anti-CD20 mAb	SC	RMS[b]	2020	2021
Ocrelizumab	Ocrevus®	Anti-CD20 mAb	IV	RMS or PPMS	2017	2018
Alemtuzumab	Lemtrada®	CD52-directed cytolytic mAb	IV	RMS[b]	2014	2013
Natalizumab	Tysabri®	Integrin receptor antagonist	IV	RMS; CD	2004	2006
Mitoxantrone[a]	Novantrone®	Synthetic antineoplastic anthracenedione	IV	RMS[c]	2000	1998
Oral medications						
Ponesimod	Ponvory®	S1P receptor modulator	Oral	RMS[b]	2021	2021
Ozanimod	Zeposia®	S1P receptor modulator	Oral	RMS[b]	2020	2020
Siponimod	Mayzent®	S1P receptor modulator	Oral	RMS[b]	2019	2020
Cladribine	Mavenclad®	Purine antimetabolite	Oral	RMS[d]	2019	2017
Dimethyl fumarate	Tecfidera®	Unknown	Oral	RMS	2013	2014
	Generics				2020	2022
Monomethyl fumarate	Bafiertam® (US)	Unknown	Oral	RMS[b]	2013	–
Diroximel fumarate	Vumerity®	Unknown	Oral	RMS[b]	2013	2021
Teriflunomide	Aubagio®	Pyrimidine synthesis inhibitor	Oral	RMS	2012	2013
Fingolimod	Gilenya®	S1P receptor modulator	Oral	RMS	2010	2011
Injectable therapies						
Glatiramer acetate	Generic	Unknown	SC	RMS[b]	2017	2016
	Glatopa® (US)			RMS	2015	–
	Copaxone®			RMS[b]	1996	2004
Pegylated IFNβ-1a	Plegridy®	Unknown	IM	RMS[b]	2021	2020
			SC		2014	2014
IFNβ-1b	Betaseron® (US) Betaferon® (EU)	Unknown	SC	RMS	1993	1995
	Extavia®	Unknown		RMS[b]	2009	2008
IFNβ-1a	Rebif®	Unknown	SC	RMS	1998	1998
	Avonex®	Unknown	IM		1996	1997

CD Crohn's disease, *EU* European Union, *IFN* interferon, *IM* intramuscular injection, *IV* intravenous, *mAb* monoclonal antibody, *PPMS* primary progressive multiple sclerosis, *RMS* relapsing forms of multiple sclerosis, *S1P* sphingosine 1-phosphate, *SC* subcutaneous injection, *US* United States

[a]Historically, mitoxantrone was used as an induction agent, but its use as a multiple sclerosis treatment has decreased in recent years due to its toxicity and the introduction of newer, better-tolerated therapies

[b]Clinically isolated syndrome, relapsing–remitting disease, and active secondary progressive disease

[c]Secondary progressive, progressive relapsing, or worsening relapsing–remitting disease

[d]Relapsing–remitting disease and active secondary progressive disease

Table 3: Disease-modifying therapies approved by the US Food and Drug Administration and the European Medicines Agency for multiple sclerosis treatment[37].

Historically, an escalation approach to disease-modifying therapies was used for newly diagnosed patients with relapsing-remitting multiple sclerosis. However, the evolution of treatment and the discovery that clinical relapses are only a small part of MS has prompted the developing concept of No Evidence of Disease Activity (NEDA). In clinical practice, the application of NEDA and the evidence for clinical benefits of early treatment with high-efficacy therapies (HETs) has led to the use of these drugs earlier in the disease course. The current line of thinking is that early treatment with high-efficacy therapies may enhance long-term clinical outcomes by minimizing the accumulation of neurological damage that occurs in the early stages of disease.

It is important to note that there is currently a lack of consensus on the classification of some DMTs as 'high-efficacy' versus 'moderate' or 'low-efficacy;' as such, DMT classifications vary between studies (this is especially true for siponimod, fingolimod, and cladribine, which are considered HETs in some studies but not in others[38,39]). Currently, the best medications that have shown better disease control in randomized phase III controlled trials compared with either active comparators of lower efficacy or placebo and are classifiable as HETs are ofatumumab, ocrelizumab, natalizumab, alemtuzumab, and cladribine[37]. Ofatumumab and ocrelizumab target the CD20 antigen present on B lymphocytes; natalizumab is an integrin receptor antagonist that blocks inflammatory cells from trespassing the BEE; alemtuzumab binds to the CD52 antigen present on mature lymphocytes; cladribine is a purine nucleoside analog that inhibits DNA synthesis limiting immune system cells expansion.

Although treatment with high-efficacy therapies with an early intensive approach has been found to be very effective and seems to result in better long-term clinical outcomes[38], these drugs are associated with a higher rate of development of side effects like hematological abnormalities, more risk of infections and malignancy.

HETs and DMTs in general, through their immunosuppressive mechanism, can lead to immune cell depletion in some patients, especially B lymphocytes for anti-CD20 antibodies leading to hypogammaglobulinemia (depletion of antibodies, which are produced by B cells); hypogammaglobulinemia is associated with a higher risk of infection, especially from encapsulated bacteria. B cells seem to repopulate the spleen

and bone marrow after the suspension of anti-CD20 therapy, so this effect is probably temporary[40]. There are other infections that could develop through the use of high-efficacy therapies, such as progressive multifocal leukoencephalopathy (PML), which derives from the reactivation of the John Cunningham virus and can lead to severe disability and death. Among the DMTs, natalizumab is by far the drug most frequently associated with JCV-associated complications; a curious fact is that the concept of "pharmacovigilance" in multiple sclerosis was born because of the registration of numerous cases of PML after the approval of natalizumab in 2000: this led to the discontinuation of its distribution in 2005 and the reapproval in 2006. PML must be diagnosed in an asymptomatic stage, so patients that assume DMTs, especially those undergoing natalizumab therapy, must be followed up over time with multiple MRI scans to achieve early recognition of the disease.

The introduction of newer, more efficacious therapies has changed the natural history of the disease, delaying the accumulation of severe neurological disabilities in most patients. Nevertheless, there are still several unsolved problems regarding treatment. Long-term studies have demonstrated that over 10 years, more than half of RRMS patients treated still experience significant disability progression, regardless of their NEDA-3 status and the presence of overt inflammatory disease activity[41]. This progression, referred to as progression independent of relapse activity (PIRA), can occur even in patients who are free of relapse and MRI activity and is becoming recognized as a key contributor to disability progression at all stages of the disease[42,43]. Furthermore, there is currently no medication that has convincingly shown the ability to promote remyelination or neuronal plasticity and reverse neurological dysfunction. Although some disease-modifying therapies (DMTs) have been associated with short-term neurological improvements, their clinical significance remains to be determined. These considerations are particularly important for individuals with aggressive MS.

1.4 Hematopoietic Stem Cell Transplant

Taking this background into consideration, intense immunosuppression followed by hematopoietic stem cell transplantation (HSCT) has emerged as a valid treatment option for aggressive MS. This approach was first investigated in animal models for severe autoimmune diseases. Reports of remarkable clinical response to HSCT performed for other conventional indications in humans provided further support for its potential benefits. In 1997, the first autologous HSCT (AHSCT) procedures for MS were carried out, and to date, over 2000 MS patients worldwide have received this treatment.

Autologous hematopoietic stem cell transplantation (AHSCT) in MS aims to eliminate self-reactive cell clones and induce self-tolerance by completely renewing the immune system. Several biological mechanisms are believed to be responsible for the resetting of the overactive immune response in MS. These include normalizing the restricted T-cell repertoire in the peripheral blood and cerebrospinal fluid, selectively reducing the encephalitogenic effector response, increasing the immunoregulatory cell subsets, and reducing pathogenic memory cells in the bone marrow niche that is believed to drive chronic inflammation[44].

AHSCT procedure

There is more than one way to obtain hematopoietic stem cells (HSCs). Firstly, the donor can be the same subject who receives the transplantation (autologous transplant) or another individual (allogeneic transplant), typically a familiar or an HLA-matched unrelated donor (MUD). The use of allogeneic hematopoietic stem cell transplantation (HSCT) for immune-mediated neurological disorders is infrequent, mainly due to the potential for severe and life-threatening adverse events. Secondly, hematopoietic stem cells can be extracted from bone marrow or peripheral blood. Peripheral blood collection is the most used method for obtaining HSCs in multiple sclerosis. Because in normal conditions there are too few HSCs in blood, this collection method requires an additional process called mobilization.

The stages necessary to perform an autologous hematopoietic stem cell transplant are:
- Pretransplant optimization

- Stem cell mobilization and collection
- Conditioning chemotherapy
- Stem cell reinfusion
- Post-transplant supportive care

AHSCT should be provided only in highly experienced centers as it is considered a high-risk procedure: conditioning regimens result in more significant immunosuppression than those used for haemato-oncological indications, and consequently, the incidence of acute reactions, viral reactivations, and infections is higher. Prior to transplantation, an extensive evaluation of the patient's fitness for transplantation is necessary, including echocardiography, pulmonary function testing, spleen ultrasonography, chest X-rays, and blood testing that screens for infections.

Mobilization and collection of peripheral blood hematopoietic stem cells (PBSCs) are achieved through the use of cyclophosphamide (CY) at a variable dosage of 1.5-4 g/m^2 associated with uromixetan and hyperhydration, followed by daily granulocyte colony-stimulating factor (G-CSF, 5–12 µg/kg/day). G-CSF acts by increasing the number of neutrophil precursors: these cells are present in bone marrow and release lytic enzymes (such as chymotrypsin) that break the CXCR4-CXCL12 bond. Disruption of this bond causes stem cells to be released into the circulatory stream. Although C-CSF could be used alone for mobilization, combination therapy is necessary to prevent clinical relapses or new MRI activity, as the isolated use of G-CSF in an autoimmune disease would lead to increased inflammatory flares[45]. Cyclophosphamide is also useful for decreasing the pool of autoreactive lymphocytes in peripheral blood, thereby reducing the likelihood that they will end up within the graft[46]. This procedure can be performed as an outpatient regimen. Peripheral hematopoietic stem cell collection is obtained through processing of the patient's blood. The patient has two venous accesses: the first allows blood collection, then leukapheresis is applied ex vivo to isolate CD34+ cells, in fact the CD34+ antigen is specific to HSC. The second access allows to reinfuse the blood from which the CD34+ cells were taken. The procedure takes a few hours, and at least 10 liters of blood are processed. This procedure can be repeated multiple times to collect a

sufficient number of cells, in MS a minimum of $4–5\times10^6$ CD34+ PBSC/kg body weight is recommended.

The conditioning phase usually occurs after 30–60 days, the patient is treated with a regimen that has the aim of eliminating the autoreactive clones in the immune system. Different conditioning protocols have been proposed and classified according to the intensity of the haemato-lymphopoietic system ablation. The EBMT[47] classifies condition regimens in high, intermediate and low intensity; intermediate regimens can be further divided into lympho-myeloablative or purely myeloablative (Table 4).

Intensity	Examples of conditioning regimens
High	Total body irradiation (TBI), cyclophosphamide and ATG, Busulfan, cyclophosphamide and ATG (BuCyATG)
Intermediate (myeloablative)	Carmustine (BiCNU) 300 mg/m^2, etoposide 800 mg/m^2, cytarabine arabinoside 800 mg/m^2 and melphalan 140 mg/m^2 (BEAM, with total doses of chemotherapy provided) and ATG ('BEAM-ATG')
Intermediate (lymphoablative/non-myeloablative)	Cyclophosphamide 200 mg/Kg and rabbit ATG (Cy-ATG)
Low	Chemotherapy only regimens e.g. single agent cyclophosphamide 100 mg/kg for mobilization and repeated 100 mg/kg for conditioning (without rituximab)

Table 4: Categorization of conditioning regimens used for autologous HSCT, with examples used in MS[47].

Following the conditioning regimen, the cryopreserved graft is thawed and the cells are infused via a central venous catheter. Administering ATG within 2-4 days after PBCS infusion can be complicated by severe allergic reactions, including fever, and premedication with corticosteroids is typically given. It's worth noting that any type of fever can potentially compromise neurological function temporarily (known as the Uhthoff phenomenon), so patients should be informed in advance about the possibility of transient neurological deterioration during AHSCT. Recovery of cell counts usually

takes 7-15 days, during which patients require supportive care such as transfusions and antibiotics. Oral prophylaxis is generally recommended for 3 months to cover fungal infections (with an azole) and for a minimum of 6 months for herpes virus (with acyclovir) and pneumocystis infection, with some units extending prophylaxis to 12 months. Monitoring for viral reactivation is crucial, and PCR-based monitoring for Epstein-Barr-Virus (EBV)/Cytomegalovirus (CMV) is recommended during the first 100 days[48].
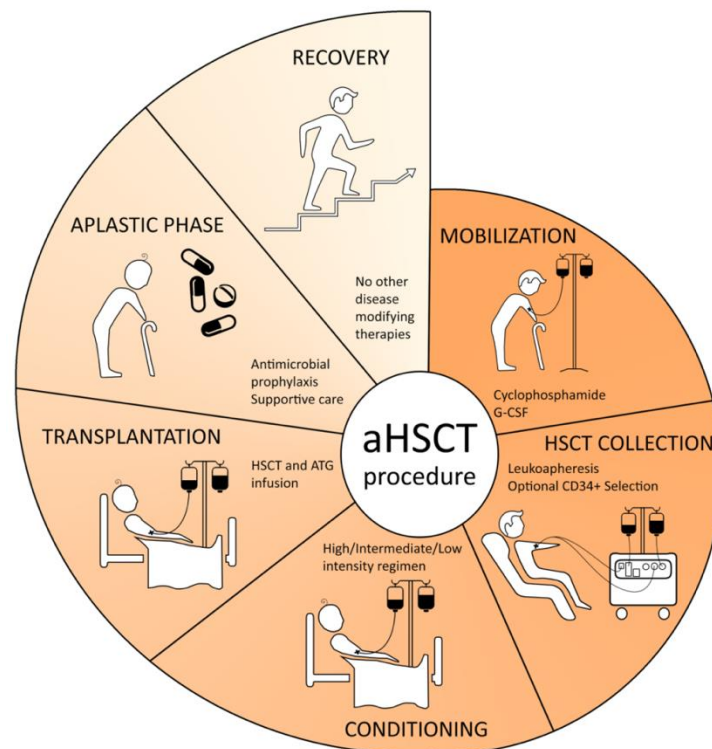
Figure 4: aHSCT procedure steps are shown in the picture.

AHSCT efficacy in MS

Numerous studies on AHSCT and MS have been done over the years, although most of these are nonrandomized, uncontrolled clinical trials, generating low levels of evidence and reporting data from heterogeneous transplant regimens and patient populations. These studies have provided insight over the years into the efficacy of AHSCT, what its risks are, and how to make a correct patient selection.

Early studies, focusing on the feasibility and safety of the procedure, included patients with advanced progressive MS. These studies showed that AHSCT was able to completely abolish inflammatory MR activity and clinical relapse, but was unable to completely prevent disease progression in long-standing inactive progressive MS[49]. According to a meta-analysis conducted by Nabizadeh et al. in 2022[50], which included all clinical studies on AHSCT in MS up until 2021, it was found that 81% of patients globally remained relapse-free 5 years after AHSCT. Additionally, only 8% of patients had new MRI lesions, resulting in an overall event-free survival of 63%.

Only two randomized controlled trials address AHSCT efficacy against DMTs. The first trial, called ASTIMS, involved 21 patients with SPMS who underwent either transplant with an intermediate myeloablative conditioning protocol or mitoxantrone. After a follow-up of 4 years, patients who received AHSCT had fewer MRI lesions and a reduced annualized relapse rate compared to the mitoxantrone group, but no difference in disability progression. The second trial, called MIST, included 110 RRMS patients who were randomized 1:1 to AHSCT with an intermediate intensity lymphoablative regimen or to the best available platform DMT. After 2 years, the EDSS in the AHSCT group was stable or improved in 94.5% of patients, and the NEDA at 5 years was 78.5% in the AHSCT group compared to 2.97% in the DMT group. The study suggests that patients with RRMS may have better long-term outcomes with intermediate-intensity lymphoablative AHSCT compared to platform DMTs.

To fully understand the long-term effectiveness of MS treatments, studies must observe patients for many years. Some studies have followed transplanted MS patients for over a decade and found that a significant percentage of patients were free of disability progression. For example, one study found that after 15 years, 44% of patients were disease progression-free[51]. Another study found that after 10 years, 65.5% of patients were free of disability worsening, with even higher rates for patients with relapsing-remitting MS[52]. These findings are supported by other extensive studies and suggest that the benefits of transplant may last for many years without the need for additional therapy.

The best intensity of the conditioning regimen for AHSCT in MS is still unknown. Intermediate intensity conditioning using CY+ATG and BEAM+ATG appears to be

the most commonly used and has shown promising results[47]. High-intensity busulfan- or total body irradiation-based conditioning protocols are rarely used due to the increased risk of death without added benefits. Low-intensity conditioning regimens are used in some countries but may not require stem cell reinfusion. The safety and efficacy of different conditioning regimens for AHSCT allow for individualized treatment based on a patient's disease course and risk profile, which is an advantage over available DMTs. No study has shown that one conditioning protocol is better than others in preventing disability progression.

Studies have found that patients with RRMS who undergo AHSCT experience sustained reductions in their EDSS disability score[52,53]. This could be due to the gradual recovery from relapses, but some studies suggest that AHSCT has a strong effect on improving neurological status beyond the first year after treatment. The chemotherapy used during AHSCT may target the chronically inflamed microenvironment that impairs remyelination and neuronal plasticity. Prevalence of improvement, or the proportion of patients who are improved at each time point, is a better indicator of meaningful changes in neurological disability than the incidence of confirmed improvement. It has been demonstrated that 50% of treated patients maintained a disability improvement 5 years after AHSCT[54].

AHSCT and MRI measurement of brain damage

According to several studies, serial MRI scans after AHSCT uniformly demonstrate a nearly complete suppression of new and gadolinium-enhancing MRI lesions, indicating a profound anti-inflammatory effect of AHSCT[48,52,55]. However, despite the anti-inflammatory effect, studies have also shown accelerated whole-brain volume loss in the first months after treatment[56,57]. The reason behind this loss of brain volume, sometimes referred to as "pseudoatrophy", is not fully understood yet, as it will be discussed in chapter 1.5 "Quantitative Volumetric Analysis". Still, potential reasons may include the cytotoxic effect of high-dose chemotherapy used for AHSCT[58,59] and pathological processes such as Wallerian degeneration following acute inflammation. Another hypothesis is that the early accelerated brain volume loss could be due to the "pseudoatrophy phenomenon", where rapid resolution of inflammatory edema causes shrinkage of MS lesions and changes in white matter and cortical microstructure[60].

The extent of gadolinium-enhancing lesions at baseline may also contribute to brain volume loss.

Different AHSCT regimens may also be associated with different levels of chemotherapy-related brain volume loss, with BEAM-based AHSCT showing relatively fewer volume changes during early follow-up than other regimens[61].

AHSCT and safety

Potential side effects of hematopoietic stem cell transplant (AHSCT) include febrile neutropenia, mucositis, electrolyte disturbances, anemia, neutropenia, low platelet count, and viral reactivation (including CMV and EBV). Fever is a common symptom during AHSCT, although it is highly unspecific and can manifest because of drug reactions, transfusion reactions, mucositis, and engraftment syndrome. Patients undergoing AHSCT are also at risk for malnutrition, which is associated with a higher risk of infections, delayed neutrophil engraftment, and prolonged hospital stay.

Despite these potential risks, the treatment-associated mortality rate of AHSCT has greatly improved in recent years due to better patient selection and the use of intermediate-intensity treatment regimens. The transplant mortality rate was 7.3% between 1995 and 2000, 1.3% between 2001 and 2007, 0.2% between 2012 and 2017, and thereafter[62]. It is important to note that while AHSCT has potential risks, it is a one-off treatment with no cumulative toxicity or treatment burden, unlike some high-efficacy disease-modifying therapies[63].

Overall, the use of AHSCT in MS was previously limited due to concerns about its toxicity, as MS is usually not a life-threatening condition. However, with more experience in transplant centers and a careful selection of patients, AHSCT can now be performed safely with a low risk of mortality from treatment.

Patient selection

Data shows that patient characteristics that are associated with better outcomes are younger age, shorter disease duration, and lower baseline disability. AHSCT should be considered early in the MS treatment algorithm, especially for patients who show

signs of aggressive MS and have a smaller therapeutic window. AHSCT may also be considered before failing a full course of DMT if aggressive characteristics of MS are present[64]

The use of AHSCT in progressive MS is still unclear, though studies have shown that AHSCT can reduce CSF markers of axonal damage and neurofilament light chain levels, slow down cognitive decline, and normalize long-term rates of cerebral gray matter and white matter atrophy, which are the main pathological features of progressive MS[61,65]. It is thought that AHSCT could target compartmentalized inflammation and pathogenic memory cells within the bone marrow niche, which are considered to drive chronic inflammation in this form of disease. However, older patients with progressive MS have an increased transplant mortality rate[66].

In conclusion, AHSCT is a highly effective treatment for MS, as it can prevent disease progression, reduce relapses, and decrease inflammation and associated CNS lesions. Although AHSCT is usually used for patients who are refractory to other DMTs and have a more aggressive disease course, studies comparing AHSCT with other DMTs have shown positive results. AHSCT has become safer with increasing knowledge and expertise in stem-cell therapy. Therefore, it is recommended to consider AHSCT as a viable treatment option for MS, while ensuring proper patient selection and transplant methods.

1.5 Chemotherapy's toxic effects on the central nervous system

As previously stated, AHSCT procedure includes several chemotherapy agents in the conditioning regimen. In recent years, the increased survival rates of cancer patients have brought attention to the potential neurotoxic effects of chemotherapy drugs, including the new targeted or immunotherapeutic agents, which are now being studied with a focus on their central nervous system (CNS) manifestations[67]. It's crucial to understand that the CNS symptoms caused by this drug may take a long time to develop, and once they do, they may not be reversible. The drug's ability to cross the blood-brain barrier is associated with the risk of developing CNS side effects.

Additionally, there are other factors that could contribute to side effect development, such as genetic predisposition.

The current hypothesis suggests that the administration of substances such as cisplatin, cytarabine, cyclophosphamide, methotrexate, fluorouracil, vinblastine, and others could lead to CNS damage through excitatory mechanisms and apoptotic cell death[68]. Table 5 contains a list of drugs that are known to cause toxicity to the central nervous system.

Agent	CNS effect
Cisplatin	Encephalopathy/Seizures/PRES
Busulfan (high dose)	Seizures
L-asparaginase	Encephalopathy/Seizures
Methotrexate (mostly high dose)	Aseptic meningitis/Acute & Subacute Encephalopathy/ Seizures/Leukoencephalopathy/PRES
Nitrosoureas	Encephalopathy/Seizures
Vincristine	Seizures/Coma/Cranial nerve mononeuropathies
Cytarabine (high dose)	Encephalopathy/Aseptic meningitis/Cerebellar toxicity/ Seizures/PRES
Cyclosporine	PRES
Cyclophosphamide	PRES
Gemcitabine	PRES
Paclitaxel	PRES
Ifosfamide (mostly high dose)	Encephalopathy/Seizures
Interferon (high dose)	Encephalopathy/Cerebellar dysfunction
Bortezomib	PRES
Thalidomide	Acute encephalopathy

Table 5: List of chemotherapy agents that are known to cause CNS toxicity and their side effects[69]. Highlighted are medications used in AHSCT conditioning protocols.

Chemotherapy-related cognitive impairment

Although the majority of chemotherapy-induced effects on the central nervous system are temporary and only result in complications for a small portion of patients, there is a possibility of a long-lasting impact on cognition for some individuals. This manifestation has been defined as "chemotherapy-related cognitive impairment" (CRCI, initially introduced by Silberfarb in 1983[70]), also known as "chemobrain" or "chemofog", and it usually manifests as permanent neurological damage and decreased cognition that manifests both clinically and through MRI changes. As this condition has a severe impact on the quality of life of cancer patients, it has been studied intensively in numerous studies[71,72] and its presence has been demonstrated as a toxic effect of many cancer treatments, such as leukemia, lymphoma, lung cancer, colorectal cancer, ovarian cancer, prostate cancer, testicular cancer. The occurrence of CRCI ranges from 17% to 70% and has even been observed to reach up to 75% in breast cancer cases[73]. Cognitive impairment may manifest during or shortly after chemotherapy or develop during the later stages of the disease. The effects of CRCI may persist for an extended period, although certain studies suggest that cognitive disorders may exhibit a tendency to improve over time[74]. Multiple pathogenetic mechanisms are thought to be implicated in the genesis of CRCI: direct toxic effects, cytokine disorders, oxidative stress, and other indirect factors all play a role in defining the condition.

Longqin et al.[75] suggested that CRCI diagnosis should be reached through self-reported cognitive impairment tools, neuropsychological tests and neuroimaging exams, especially magnetic resonance imaging (MRI) and positron emission tomography (PET). After undergoing chemotherapy, patients showed changes in brain structures as seen through MRI studies. These changes were observed in the insula, bilateral hippocampal gyrus, and left anterior cingulate cortex[75]. Overall, there was a decrease in overall gray matter volume and damage to white matter integrity. Diffusion tensor imaging (DTI) studies[76,77] have shown that breast cancer patients who receive adjuvant chemotherapy can develop impaired white matter integrity; additionally, this finding is associated with decreased cognitive functions such as attention and verbal memory.

To summarize, although chemotherapy-induced effects on the central nervous system typically don't last long and only impact a small number of patients, it's crucial to acknowledge that some individuals may experience long-term impacts on their cognitive function. Advances in research and treatment can help mitigate the risk of chemotherapy-related cognitive impairment and improve the quality of life for those affected.

1.6 MRI role in Multiple Sclerosis, Quantitative Brain MRI and advanced MRI sequences

Magnetic resonance imaging (MRI) is a non-invasive diagnostic tool used to visualize the CNS. It is considered the most sensitive and specific imaging technique for the detection of MS. MRI is used to identify and characterize the lesions that are typically seen in patients with MS, including both acute Gd+ and chronic lesions.

Magnetic Resonance has become an essential exam and fulfills multiple roles in MS: it is crucial to establish a diagnosis with MAGNIMS or McDonald criteria, but it is also useful in differential diagnosis, treatment, and prognosis. As the number of available disease-modifying therapies for MS continues to grow, MRI has become a critical tool for evaluating and tracking treatment effectiveness. Furthermore, the role of MRI in patients with MS has broadened to include safety monitoring, such as detecting CNS-related adverse events, both infectious and non-infectious. As a final point, magnetic resonance imaging in multiple sclerosis is crucial in research to establish the efficacy, safety, and adverse effects of new therapeutic approaches such as AHSCT.

The most common techniques used in MRI for MS include T1-weighted, T2-weighted, and gadolinium-enhanced imaging. Standard MRI protocols allow for the visualization of different aspects of MS pathology, including the presence of lesions, the degree of inflammation, and the extent of gross demyelination. In clinical practice, these sequences help establish a diagnosis and manage the disease but carry some limitations. For example, there are widespread white matter abnormalities that appear normal on T2- and T1-weighted images; additionally, gray matter lesions and brain

atrophy are usually not studied with traditional MRI imaging. To overcome these limitations, quantitative MRI and advanced imaging techniques have been developed, which provide a more sensitive and specific assessment of MS tissue injury and can provide insight into pathological mechanisms giving a better understanding of MS pathophysiology.

Quantitative MRI

When performing imaging studies, the term "quantitative" is frequently used to describe the measurement of numerical values of signal intensities in addition to the conventional "qualitative" visual inspection of the images.

Conventional MRI can only reveal gross morphological abnormalities or focal abnormalities resulting in regional differences in signal intensities. It relies on differences in contrast between areas presumed to be affected and areas deemed normal. As a result, conventional "qualitative" MRI is inherently insensitive to subtle global changes in the brain, such as brain atrophy.

Quantitative MRI techniques, including relaxometry, myelin imaging, magnetization transfer, diffusion MRI, quantitative susceptibility mapping, and perfusion MRI, provide valuable information about disease mechanisms in patients with multiple sclerosis. These techniques complement conventional MRI methods to enhance our understanding of the disease.

Quantitative MRI provides information about normal-appearing tissue pathology, multiple sclerosis lesion heterogeneity, remyelination, and blood–brain barrier disruption[78].

The concept of brain atrophy in MS

The term "atrophy" describes a volumetric decrease of the brain or some of its regions. In multiple sclerosis, this process is believed to derive from the resolution of inflammation and neuro-axonal loss. Brain atrophy, measured in MRI as BLV (brain volume loss), is a phenomenon that was thought to be typical of late-stage MS, but can also appear as early as CIS, progresses faster than it does in healthy adults, and is the best predictor of future disability, physical and cognitive[79]. Furthermore, increased

brain volume loss (BVL) has been correlated with disability progression, independent of the number of previous relapses or the T2 lesion load in RRMS[80].

Whole brain atrophy in multiple sclerosis occurs at rates of 0,5–1,5% per year, and faster rates could be seen in the progressive phases of the disease and in the deep grey matter structures[81].

Multiple substrates can be responsible for brain volume loss in MS, including[60]:

- Loss of volume within white matter (WM) lesions due to the loss of myelin, oligodendrocytes and axons and the contraction of astrocyte volume occurring during lesion maturation
- Neuronal and glial loss in cortical grey matter (GM) lesions
- Volume loss in normal-appearing brain tissue

The importance of brain atrophy in MS has grown over the years. It is now considered an important biomarker and endpoint in studies to measure treatment efficacy and toxicity. In clinical practice, volumetric MRI-based measurements are used to evaluate the efficacy of disease-modifying treatments and high-efficacy therapies.

As previously said, AHSCT has been associated with faster rates of BVL in the first months after the procedure, although the nature of this phenomenon has not yet been characterized. Different hypotheses have been made, including chemo-toxicity derived from the conditioning regimen, other pathological processes like Wallerian degeneration, and "pseudoatrophy".

Pseudoatrophy is defined as a loss of brain volume derived from the resolution of neuroinflammation and it is not correlated with irreversible tissue loss. The pathophysiological mechanism underlying pseudoatrophy is still unknown, although it is thought to derive from the resolution of brain edema and fluid shifts. It is believed to be the most important cause of brain volume loss, especially in the early months after therapy initiation[82]. In this context, significant treatment effects were seen in some trials[82,83] only after the initial year of treatment, when it is assumed that the pseudo-atrophy effect has either subsided or become less prominent. Contrary to what was previously thought, studies have demonstrated that pseudoatrophy occurs primarily in gray matter compartments and affects white matter to a lesser extent[84].

Previous studies have demonstrated that patients with more Gd+ active lesions tend to have a higher brain volume loss in comparison to those without gadolinium-enhancing

lesions after anti-inflammatory treatment initiation[84,85]. This phenomenon has also been observed in patients treated with AHSCT[61].

Pseudoatrophy is known to be involved in the early stages of MS treatment, but little is known about its long-term presence. The existence of pseudoatrophy poses a problem in characterizing BVL in MS, and advanced MRI techniques are needed to distinguish it from "true" atrophy. Another problem is that no specific brain regions in which to study pseudoatrophy have yet been identified.

Diffusion Tensor Imaging (DTI)

Diffusion tensor imaging (DTI) is an advanced MRI technique that measures the movement of water molecules in the brain. In MS, DTI can be used to assess the damage to myelin and axons in the brain's white matter tracts. By analyzing the diffusion of water molecules, DTI can detect changes in the structural integrity of the white matter tracts, which are untraceable with standard MRI protocols (normal appearing white matter, or NAWM). In fact, DTI-based measurements, such as fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD), and axial diffusivity (AD), appear to offer greater specificity in detecting demyelination and axonal damage than conventional MRI techniques[86]. DTI has proven to be a sensitive tool in monitoring diffuse abnormalities responsible for disability accumulation in MS[87].

Aim of the study

It's already been established (Chapter 1.2) that some patients can develop aggressive forms of multiple sclerosis. Although there is no consensus on the definition of this condition, this group tends to develop relapses, MRI lesions or disability progression despite the use of disease-modifying treatments (DMT).

Autologous hematopoietic stem cell transplantation (AHSCT) has proven to be an effective treatment for aggressive multiple sclerosis as it has been shown to reduce the rate of disease progression in this population[48,52]. This procedure has been extensively studied for its clinical impact, but there is limited knowledge about its effects on MRI biomarkers in the medium and long term. While few studies have indicated that atrophy rates decrease after long-term transplantation, there is evidence of accelerated atrophy in the early stages post-transplantation[56,57,88–90]. Some argue that this accelerated atrophy could be due to a direct neurotoxic effect of chemotherapy[58]. Indeed, chemotherapy is known to be associated with direct toxic effects on the central nervous system, and advances in the field of oncology have provided a better understanding of its mechanism. As discussed previously, the phenomenon of "chemotherapy-related cognitive impairment", or "chemofog", has been correlated with the development of an increased rate of brain atrophy[75], similar to what happens after the AHSCT procedure.

On the other hand, others argue that the pathological substrate underlying brain atrophy after transplantation could be pseudoatrophy related to the resolution of inflammation and edema. In fact, pseudoatrophy is often implicated as one of the main mechanisms of brain volume loss in the early stages after the initiation of high-efficacy treatments. It is possible that both phenomena coexist in the context of transplantation, as new data suggest that a key role could be played by the conditioning regimen utilized. In fact, the HALT MS study, in which a BEAM regimen was used, saw less accelerated atrophy in comparison to patients who underwent a busulfan-based conditioning regimen[61].

One way to address this problem is by analyzing volumetrics and diffusion data simultaneously, which could provide new valuable insights. Diffusion MRI is

particularly useful for examining the microstructural integrity of normal-appearing tissue and detecting extracellular water content, making it a useful metric to consider. The impact of IA/HSCT treatment on both gray matter (GM) and white matter (WM) and how they contribute to brain shrinkage is not fully understood. By understanding their roles, we can gain a better understanding of the cause of treatment-related brain shrinkage. If GM volume loss is accelerated, it may indicate that the treatment has primarily a neurotoxic effect, causing irreparable damage to GM tissues. On the other hand, if WM volume loss is accelerated, it may suggest degeneration of WM components like axons or myelin due to the treatment, as well as irreparable damage to WM tissues.

Against this background, the aims of my thesis were:
- To assess the rates of global, white matter and gray matter atrophy in the short and long term in a cohort of patients with aggressive MS undergoing intermediate-intensity transplantation
- To assess the integrity of normal-appearing white matter at those same timepoints to investigate changes in extracellular water content and microstructural integrity

Materials and methods

3.1 Participants

This study was an observational, prospective study aimed at the definition of AHSCT procedure effects on the brain, collecting data from consecutive patients transplanted from the MS center of Genoa and Turin, from 2006 to 2019.

All patients were diagnosed with multiple sclerosis according to the 2005 and 2010 McDonald Criteria. All patients presented manifestations of disease compatible with the definition of aggressive MS.

Written informed consent was obtained from all patients. All participants provided consent to use their medical history for publication. This retrospective study was approved by the ethical standards committee of the coordinating center.

3.2 Conditioning regimen and transplant care

In July of 1998, five Italian neurologic teams initiated a phase I/II trial on the use of AHSCT in MS, in conjunction with the Italian Cooperative Group for Bone Marrow and Blood Transplantation (GITMO). Following this, other Italian MS centers established similar transplant programs for MS patients, modeled after those developed by the leading haemato-neurological centers in Florence and Genoa. Although there are no formal guidelines for patient selection for AHSCT, all treated patients had aggressive MS characterized by severe relapses, MRI inflammatory activity, or accelerated neurological disability despite active treatment. Treatment followed the European Group for Blood and Marrow Transplantation (EBMT) guidelines, and patients were selected at the treating physician's discretion and with approval from the local Ethics Committee.

Peripheral hematopoietic stem cells mobilization was obtained with cyclophosphamide (CY) (4 or 2g/m2 iv) and filgrastim (5-10 g/kg/day sc). To collect PBSCs, a leuko-apheresis procedure was utilized. The resulting graft, containing 3-8x10^6 CD34+ cells/kg, was cryopreserved in its unmanipulated state.

Different conditioning protocols were used to obtain deep immunosuppression, according to the center's experience and preference. 17 patients received a BEAM + ATG regimen, consisting of BCNU (carmustine, 300 mg/m^2 at day -6), cytosine-arabinoside (200 mg/m^2) and etoposide (200 mg/m^2) from day -5 to day -2 and melphalan (140 mg/m^2) at day -1, followed by rabbit anti-thymocyte globulin (ATG) (3.75-5 mg/kg/day) at days +1 and +2;

1 patient received cyclophosphamide (CY) + ATG regimen containing CY (60 mg/kg at day -3 and-2) followed by rabbit ATG (3.75 mg/kg/d at day +1 and +2). The center's protocols involved using Acyclovir and Sulfamethoxazole-Trimethoprim to prevent herpes and pneumocystis jirovecii infections. Patients who underwent AHSCT did not receive immune-based treatments unless they showed signs of clinical relapse, new lesions on MRI or EDSS progression, as decided by the treating neurologist.

3.2 MRI acquisition

All patients underwent MRI scan with a 1.5 MRI magnet. The MRI protocol consisted of: (a) high-resolution fast-spoiled-gradient-echo (FSPGR) 3D T1-weighted sequence; (b) 3D Fluid attenuated inversion recovery (FLAIR) sequence.

Axial single-shot spin-echo echo-planar diffusion tensor imaging (DTI) sequences were obtained for 10 patients.

3.3 MRI analysis

For every MRI scan, FLAIR lesion masks were obtained using Sinlab (SienaImaging, Italy) and checked by a trained neurologist and manually corrected if necessary. To correct for non-uniformity or variations in the magnetic field generated by the machine, NU correction was performed for all scans. The T1 lesion mask was used to inpaint corresponding 3D T1 images, using FSL. Subsequently, longitudinal brain volumes were obtained using CAT12 of the SPM12 software. An example of CAT-12 segmentation is shown in Figure 5.

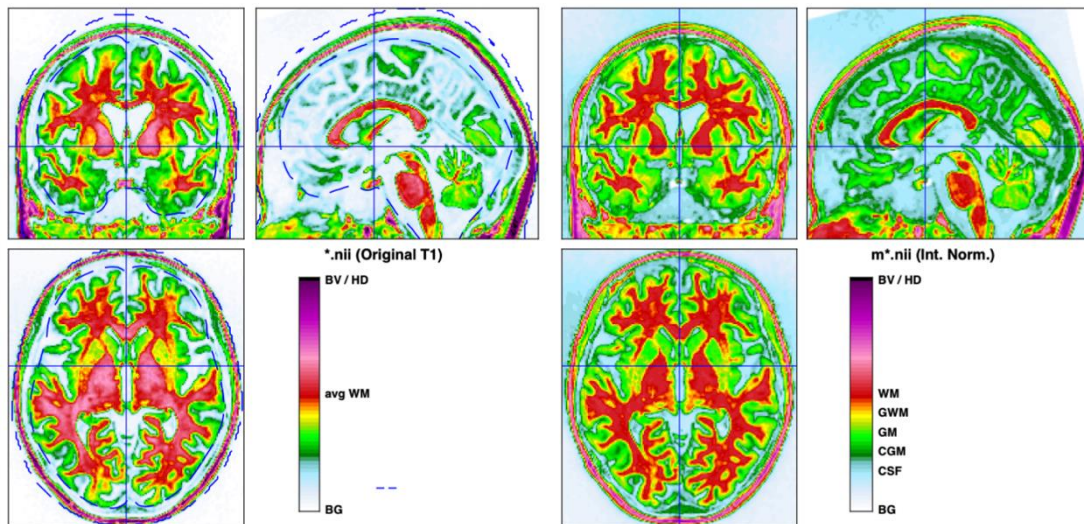**Figure 5**: CAT-12 segmentation was used to calculate total parenchymal volumes, WM and GM volumes.

DTI data processing was obtained using FMRIB's Diffusion Toolbox, part of FSL. The images were initially corrected for any eddy current distortions and were then processed through principal component analysis denoising. After that, the standard DTI maps of fractional anisotropy (FA) were obtained. To investigate WM

microstructure integrity after AHSCT, we focused on brain regions rich in myelin. Since corpus callosum (CC) has been recently proposed as a biomarker for clinical trials looking at myelin integrity, we extracted FA values from CC using an atlas-based approach (JHU-DTI-based-Atlas) in each timepoint. Figure 6 shows an example of creation of a CC atlas-based mask used for obtaining FA values.
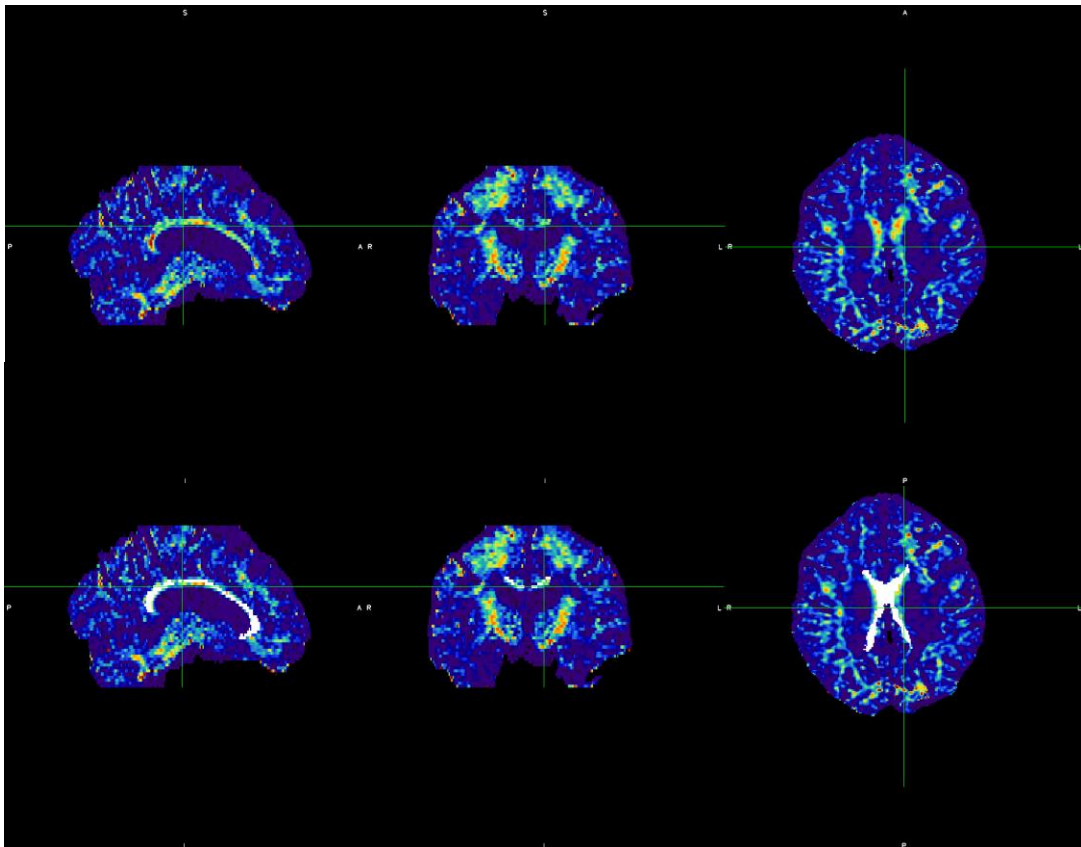
Figure 6: Corpus callosum (CC) masks (shown in white) were created in order to obtain FA values.

3.4 Statistical analysis

Statistical analyses for all clinical and MRI data were performed using SPSS 23 software (IBM; version 23.0). Descriptive results have been reported as mean with standard deviation (SD) or median with range. Shapiro–Wilk test and visual inspection of the histograms have been performed to evaluate variable distribution. Repeated measures analysis of variance (RM-ANOVA) was performed in order to analyze the evolution of brain volumes and FA metrics over time. Two-sided p-values<0.05 were be considered significant.

Results

Twenty-one subjects were initially included in the study. For 3 patients, 3D-T1 sequences were obtained after gadolinium (Gd) contrast injection and were not suited for high-quality tissue segmentation. One further subject had to be removed for excessive head motion which would have impaired correct estimation of brain volumes. The final cohort of patients consisted of 17 subjects. Table 6 reports main demographic and clinical characteristics.

Number of subjects = 17, transplanted between 06/2010 and 04/2019 with at least 12 months of Quantitative MRI follow-up. 15 patients were followed in Genoa, 2 patients in Turin. 16 patients received BEAM+ATG as conditioning regimen, 1 patient received CY+ATG.

Mean age at mobilization [range]	25-46; SD 6.3
Sex, Female:Male	12:5
EDSS [Median]	5 (1.5-8)
Number of prior therapies [median]	3 (1-6)
Number of relapses in the previous 12 months[mean]	3.1; SD 2.5
Presence of at least 1 Gadolinium+ lesion	13/17 subjects
Number of Gadolinium+ lesions [mean]	3.0; SD 5.5
Baseline T2 lesion volume [mean]	26.4 ml; SD 28.5
Baseline MRI acquisition [mean]	60 days before AHSCT; SD 83

Table 6: Baseline demographic table.

4.1 Brain tissues segmentation

For brain tissues volumetric analyses, fourteen subjects had 3 MRI scans available, for a mean total follow-up of 626 days (SD 445; median 466, range 336-2093). For 8 subjects, 6 MRI scans were available, resulting in a mean follow-up of 1794 days (SD 617; median 1586, range 1274-3392).

Figure 7 shows a plot chart of parenchymal values for the first 3 timepoints and for the longer follow-up of 6 timepoints. Table 7 reports parenchymal values for these timeframes.
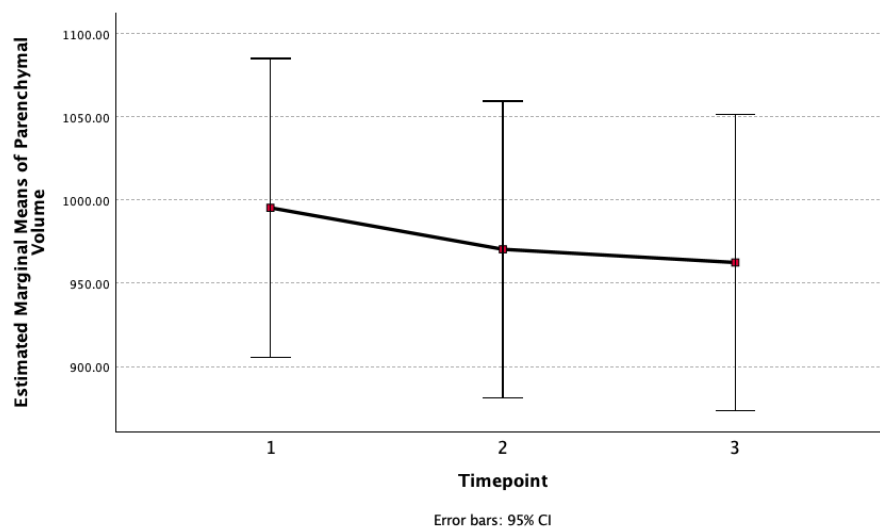

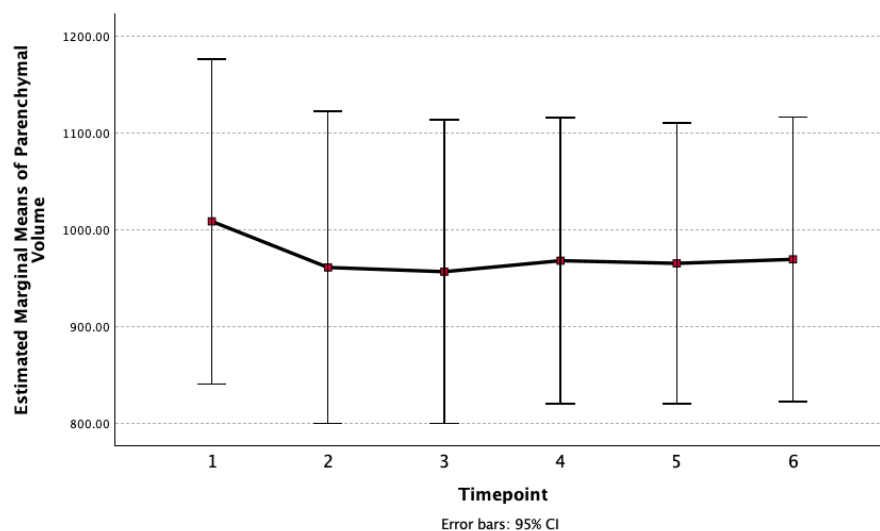**Figure 7(a)**: Plot chart of parenchymal volume values in 3 timepoints.

Figure 7(b): Plot chart of parenchymal volume values in 6 timepoints.

Early Parenchymal Volumes

	Mean	Std. Deviation	N
Parenchymal base	995.3571	155.38221	14
Parenchymal 1	970.4286	154.35111	14
Parenchymal 2	962.5000	154.03084	14

Table 7(a): Shown are the parenchymal volume values for 3 timepoints.

Long-term Parenchymal Volumes

	Mean	Std. Deviation	N
Parenchymal base	1008.5000	200.68810	8
Parenchymal 1	960.8750	193.08950	8
Parenchymal 2	956.5000	187.70874	8
Parenchymal 3	967.8750	176.86511	8
Parenchymal 4	965.1250	173.41975	8
Parenchymal 5	969.2500	175.84551	8

Table 7(b): Shown are the parenchymal volume values for 6 timepoints.

Gray matter (GM) and white matter (WM) volumes were obtained through segmentation and were measured in parallel with total parenchymal volumes. The early reduction in WM volumes was significant ($p=0.003$), while GM results were non-significant. Figure 8 and 9 show a plot chart of the volume trend change for WM and GM, table 8 and 9 report volume values for these markers.

Figure 8(a): Plot chart of WM volume values in 3 timepoints.
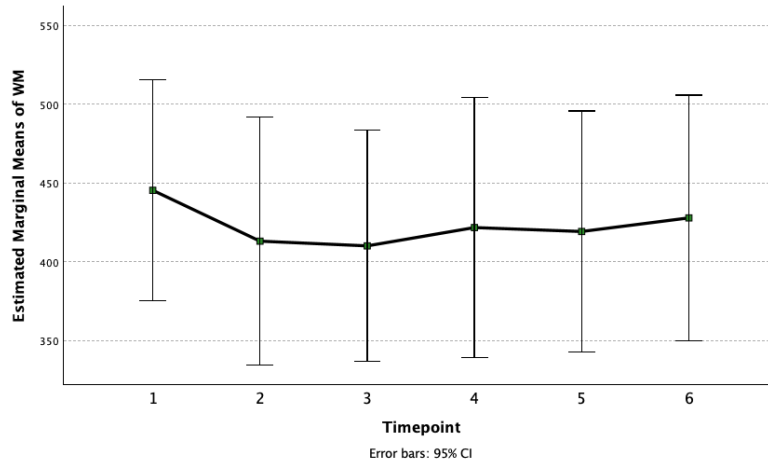
Figure 8(b): Plot chart of WM volume values in 6 timepoints.

Early WM Volumes

	Mean	Std. Deviation	N
WM baseline	440.71	65.872	14
WM 1	420.14	76.948	14
WM 2	415.93	73.600	14

Table 8(a): WM volume values for 3 timepoints.

Long-term WM Volumes

	Mean	Std. Deviation	N
WM baseline	445.37	83.991	8
WM 1	413.13	94.225	8
WM 2	410.13	87.728	8
WM 3	421.75	98.536	8
WM 4	419.25	91.380	8
WM 5	427.88	93.087	8

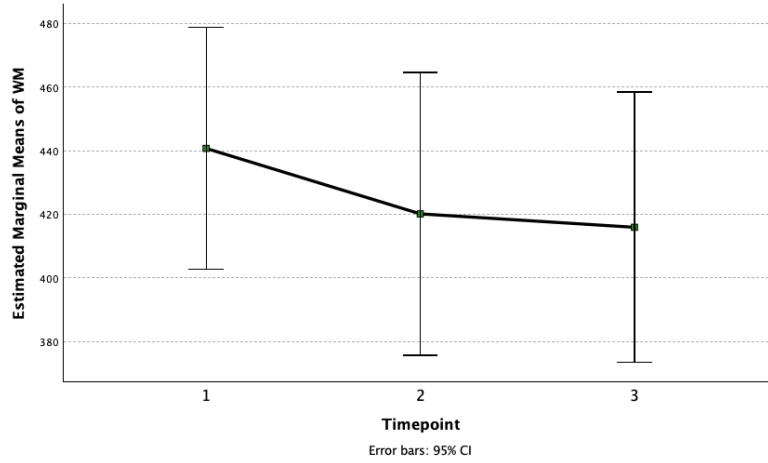**Table 8(b)**: WM volume values for 6 timepoints.
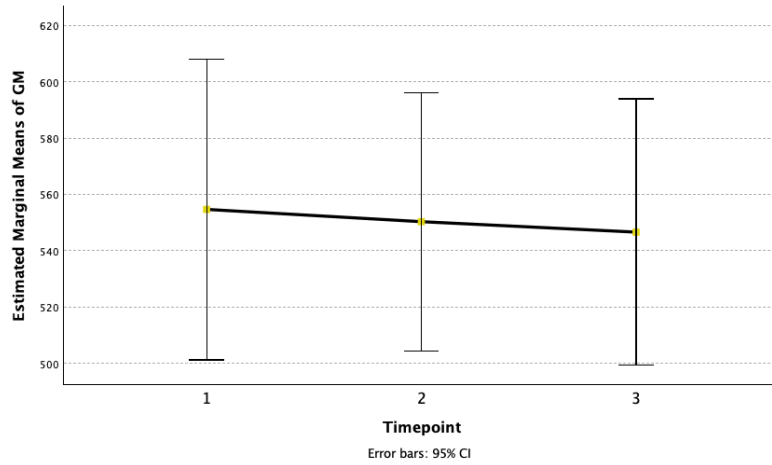
Figure 9(a): Plot chart of GM volume values in 3 timepoints.
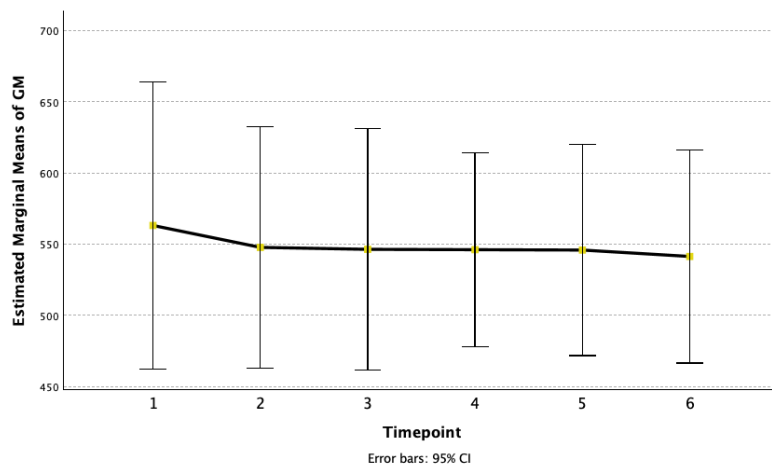
Figure 9(b): Plot chart of GM volume values in 6 timepoints.

Early GM Volumes

	Mean	Std. Deviation	N
GM baseline	554.64	92.536	14
GM 1	550.29	79.425	14
GM 2	546.57	81.943	14

Table 9(a): GM volume values for 3 timepoints.

Long-term GM Volumes

	Mean	Std. Deviation	N
GM baseline	563.13	120.545	8
GM 1	547.75	101.336	8
GM 2	546.38	101.554	8
GM 3	546.13	81.260	8
GM 4	545.88	88.599	8
GM 5	541.38	89.478	8

Table 9(b): GM volume values for 6 timepoints.

4.2 Diffusion Tensor Imaging

To investigate the impact of AHSCT on WM microstructure, we analyzed fractional anisotropy (FA) in the corpus callosum. For 3 subjects DWI images were not available, while for 4 subjects they were of insufficient quality (n=2 number of DTI directions <30, n=2 excessive head motion) for the analysis. We analyzed 10 subjects with 3 MRI timepoints (626 days, SD 445), while only 5 subjects had 6 timepoints (1794 days, SD 617). Figure 10 shows FA plot charts that were obtained for 3 timepoints and 6 timepoints, table 10 reports FA diffusivity values.

Figure 10(a): Plot chart of FA diffusivity values for 3 timepoints.

Figure 10(b): Plot chart of FA diffusivity values for 6 timepoints

Early FA Values

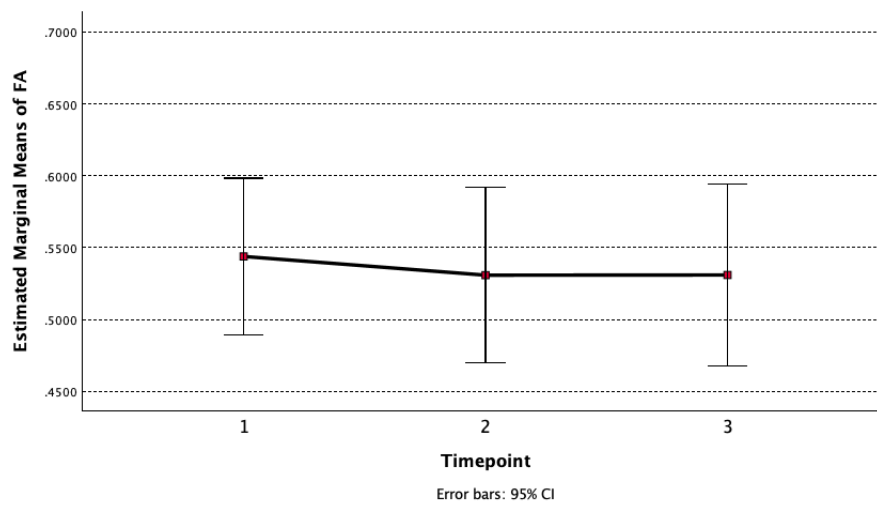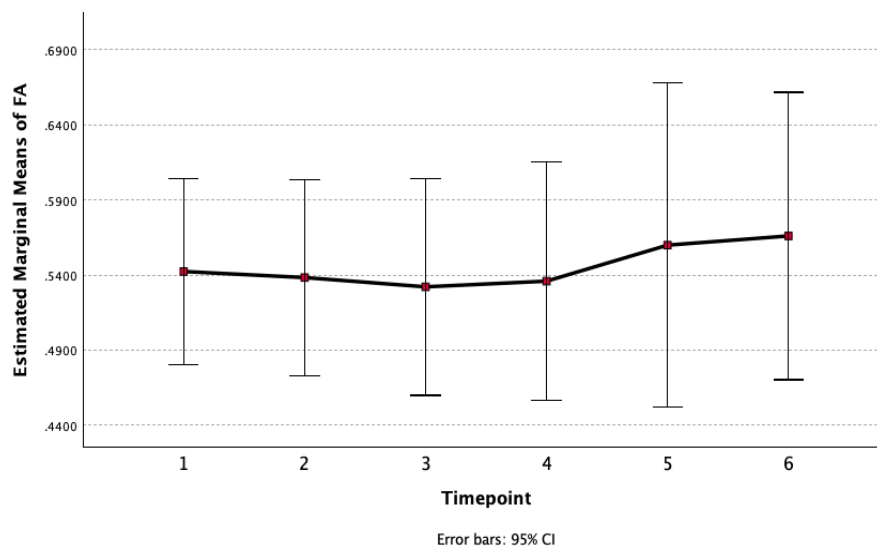|	Mean	Std. Deviation	N
FA CC mean baseline	.5438765	.0762611	10
FA CC mean 1	.5308613	.0854964	10
FA CC mean 2	.5309872	.0887437	10

Table 10(a): FA diffusivity values for 3 timepoints.

Long-Term FA Values

	Mean	Std. Deviation	N
FA CC mean baseline	.5423482	.0497939	5
FA CC mean 1	.5383102	.0525735	5
FA CC mean 2	.5321394	.0581109	5
FA CC mean 3	.5359214	.0640450	5
FA CC mean 4	.5598994	.0870084	5
FA CC mean 5	.5660294	.0771524	5

Table 10(b): FA diffusivity values for 6 timepoints.

Discussion

Our study assessed structural and diffusion brain metrics of 17 patients who underwent autologous hematopoietic stem cell transplantation as treatment for aggressive MS. The aim was to investigate the impact of AHSCT on brain volume and to explore the microstructural changes caused by the procedure.

5.1 Early structural changes

We found a significant reduction of total brain volume (TBV) in the early stages after transplantation (6-12 months), which was mainly driven by a significant reduction in WM volumes. On the contrary, GM volumes did not reduce significantly and appeared to be stable throughout follow-up. These results expand previous works, showing that AHSCT is associated with rapid brain atrophy which interests mostly the WM compartment. Of interest, in the same timepoints where significant reduction of TBV is seen, fractional anisotropy shows only minor changes. This is the first study

assessing diffusion-derived parameters of WM after AHSCT in MS, thus providing clues on tissues microstructural integrity.

The main debate on the pathological substrate of BVL after AHSCT sees as possible explanations (i) neurotoxicity from the chemotherapy used in the conditioning regimen and (ii) pseudoatrophy, a rapid reduction in edema and inflammation of the brain following immunotherapy.

In the former case, one would expect to observe a total reduction in parenchyma associated with a concomitant reduction primarily in gray matter, with a worsening of DTI measures in the total WM. Indeed, previous studies conducted in an attempt to understand the effect of neurotoxicity, found that chemotherapy results in a profound alteration in diffusion signaling[76,77]. Vice versa, if the pseudoatrophy hypothesis were true, the reduction in total volume would result primarily from a loss of volume in the white matter, with stable or ameliorated DTI metrics in the WM.

Globally, the results of our study strongly support the hypothesis that the main driver of accelerated brain atrophy observed in transplanted patients is due to pseudoatrophy mechanisms. Indeed, WM compartment seems to be the main site of volume reduction, without signs of ongoing tissue damage at diffusion imaging. Our results are in line with some, but not all, studies assessing brain volumes changes after AHSCT. An important factor that could explain these contradictory results is the conditioning regimen used during AHSCT. Our patients underwent intermediate-intensity condition regimens (17 patients with BEAN+ATG, 1 patient with cyclophosphamide + ATG), which are known to be associated with fewer CNS adverse events compared to high-intensity conditioning regimens containing busulfan. This could explain why our results are in line with data from the HALT-MS study[61] which used the same intermediate-intensity conditioning regimen, but are in contrast with the Canadian trial which used a busulfan-based conditioning regimen[58].

Although it is true that neurotoxicity and pseudoatrophy could co-exist, and it is true that some degree of neurotoxicity may be present in the aftermath of transplantation, it is possible that this effect is clinically relevant only when high-dose regimens are used, whereas the main cause of BVL in patients going on AHSCT with intermediate intensity regimens appears to be decreased edema and brain inflammation.

5.2 Long-term effects of AHSCT

As second aim of the study, we wanted to analyze MRI data after several years after AHSCT, to assess if the effects of the procedure last over time. Indeed, AHSCT is a one-shot therapy with an early profound immunosuppressive effect, but its long-lasting effects on MRI are not well characterized. We had the opportunity to analyzed brain volume changes and diffusion data up to 5 years from AHSCT, and we found that brain atrophy, after the initial acceleration discussed above, tends to slow down until it reaches a level that is comparable to normal aging. Similarly, FA remained stable over time, suggesting preserved brain tissue integrity up to 5 years from AHSCT. These results are quite impressive, considering that patients who underwent AHSCT were affected by particularly aggressive MS and in whom accelerated brain atrophy is expected.

To conclude, while we confirmed that AHSCT causes an acceleration of brain atrophy, and we suggested that this phenomenon should be primarily attributed to a resolution of edema and inflammation, this study also demonstrated again the long-lasting, profound anti-inflammatory effect of AHSCT as a treatment for aggressive MS by showing the relative normalization of total brain volume loss trends for these patients.

5.3 Limitations

This study is not without limitations. First, 2 patients underwent brain MRI using a different MRI scanner and this could have partly biased our results. However, for volumetric analyses, all 3D-T1 were of high quality isotropic $1x1x1$ mm^3 resolution. Second, it is important to note that the diffusion data derives from a small cohort of patients (n=10), therefore our statistical analysis must be considered as descriptive. Moreover, recent data showed that pseudoatrophy mechanisms could involve GM in an extensive way, thus limiting the utility of regional volumetric analysis in distinguishing between neurotoxicity and pseudoatrophy. Lastly, the effect of AHSCT on individual MS lesions could be add important information on the anti-inflammatory effect of AHSCT and needs to be evaluated in a specific study.

References

1.   The Multiple Sclerosis International Federation. Atlas 3rd Edition Epidemiology report.

2.   AISM (Associazione Italiana Sclerosi Multipla). Barometro della Sclerosi Multipla 2022.

3.   Koch-Henriksen N, Sørensen PS. The changing demographic pattern of multiple sclerosis epidemiology. *Lancet Neurol*. 2010;9(5):520-532. doi:10.1016/S1474-4422(10)70064-8

4.   Puthenparampil M, Perini P, Bergamaschi R, Capobianco M, Filippi M, Gallo P. Multiple sclerosis epidemiological trends in Italy highlight the environmental risk factors. *J Neurol*. 2022;269(4):1817-1824. doi:10.1007/s00415-021-10782-5

5.   Dobson R, Giovannoni G. Multiple sclerosis - a review. *Eur J Neurol*. 2019;26(1):27-40. doi:10.1111/ene.13819

6.   Brownlee WJ, Hardy TA, Fazekas F, Miller DH. Diagnosis of multiple sclerosis: progress and challenges. *The Lancet*. 2017;389(10076):1336-1346. doi:10.1016/S0140-6736(16)30959-X

7.   Marrie RA. Environmental risk factors in multiple sclerosis aetiology. *Lancet Neurol*. 2004;3(12):709-718. doi:10.1016/S1474-4422(04)00933-0

8.   Libbey JE, McCoy LL, Fujinami RS. Molecular mimicry in multiple sclerosis. *Int Rev Neurobiol*. 2007;79:127-147. doi:10.1016/S0074-7742(07)79006-2

9.   Babaloo Z, Aliparasti MR, Babaiea F, Almasi S, Baradaran B, Farhoudi M. The role of Th17 cells in patients with relapsing-remitting multiple sclerosis: interleukin-17A and interleukin-17F serum levels. *Immunol Lett*. 2015;164(2):76-80. doi:10.1016/j.imlet.2015.01.001

10.   Audoin B, Zaaraoui W, Reuter F, et al. Atrophy mainly affects the limbic system and the deep grey matter at the first stage of multiple sclerosis. *J Neurol Neurosurg Psychiatry*. 2010;81(6):690-695. doi:10.1136/jnnp.2009.188748

11.   Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. *N Engl J Med*. 2018;378(2):169-180. doi:10.1056/NEJMra1401483

12.   J L, K B, V J, et al. Defining secondary progressive multiple sclerosis. *Brain J Neurol*. 2016;139(Pt 9). doi:10.1093/brain/aww173

13.   Lublin FD, Reingold SC, Cohen JA, et al. Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology*. 2014;83(3):278-286. doi:10.1212/WNL.0000000000000560

14.   Bi G, Cm H, Sa G, et al. Cognitive dysfunction in patients with clinically isolated syndromes or newly diagnosed multiple sclerosis. *Mult Scler Houndmills Basingstoke Engl*. 2007;13(8). doi:10.1177/1352458507077943

15.   *Harrison's Principles of Internal Medicine*. Fourteenth edition / [editors, Anthony S. Fauci ... [and others]. New York : McGraw-Hill, Health Professions Division, [1998] ©1998; 1998. https://search.library.wisc.edu/catalog/999808448102121

16.   Saade C, Bou-Fakhredin R, Yousem DM, Asmar K, Naffaa L, El-Merhi F. Gadolinium and Multiple Sclerosis: Vessels, Barriers of the Brain, and Glymphatics. *AJNR Am J Neuroradiol*. 2018;39(12):2168-2176. doi:10.3174/ajnr.A5773

17.   van der Vuurst de Vries RM, Mescheriakova JY, Wong YYM, et al. Application of the 2017 Revised McDonald Criteria for Multiple Sclerosis to Patients With a Typical Clinically Isolated Syndrome. *JAMA Neurol*. 2018;75(11):1392-1398. doi:10.1001/jamaneurol.2018.2160

18.   Vattoth S, Kadam GH, Gaddikeri S. Revised McDonald Criteria, MAGNIMS Consensus and Other Relevant Guidelines for Diagnosis and Follow Up of MS: What Radiologists Need to Know? *Curr Probl Diagn Radiol*. 2021;50(3):389-400. doi:10.1067/j.cpradiol.2020.06.006

19.   Havrdova E, Galetta S, Hutchinson M, et al. Effect of natalizumab on clinical and radiological disease activity in multiple sclerosis: a retrospective analysis of the Natalizumab Safety and Efficacy in Relapsing-Remitting Multiple Sclerosis (AFFIRM) study. *Lancet Neurol*. 2009;8(3):254-260. doi:10.1016/S1474-4422(09)70021-3

20.   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 Houndmills Basingstoke Engl*. 2016;22(10):1297-1305. doi:10.1177/1352458515616701

21.   Iacobaeus E, Arrambide G, Amato MP, et al. Aggressive multiple sclerosis (1): Towards a definition of the phenotype. *Mult Scler Houndmills Basingstoke Engl*.

2020;26(9):1031-1044. doi:10.1177/1352458520925369

22.   Menon S, Shirani A, Zhao Y, et al. Characterising aggressive multiple sclerosis. *J Neurol Neurosurg Psychiatry*. 2013;84(11):1192-1198. doi:10.1136/jnnp-2013-304951

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):907-911. doi:10.1212/wnl.46.4.907

24.   Gholipour T, Healy B, Baruch NF, Weiner HL, Chitnis T. Demographic and clinical characteristics of malignant multiple sclerosis. *Neurology*. 2011;76(23):1996-2001. doi:10.1212/WNL.0b013e31821e559d

25.   Menon S, Zhu F, Shirani A, Oger J, Freedman MS, Tremlett H. Disability progression in aggressive multiple sclerosis. *Mult Scler Houndmills Basingstoke Engl*. 2017;23(3):456-463. doi:10.1177/1352458516653273

26.   Rush CA, MacLean HJ, Freedman MS. Aggressive multiple sclerosis: proposed definition and treatment algorithm. *Nat Rev Neurol*. 2015;11(7):379-389. doi:10.1038/nrneurol.2015.85

27.   Saccardi R, Freedman MS, Sormani MP, et al. A prospective, randomized, controlled trial of autologous haematopoietic stem cell transplantation for aggressive multiple sclerosis: a position paper. *Mult Scler Houndmills Basingstoke Engl*. 2012;18(6):825-834. doi:10.1177/1352458512438454

28.   Pérez-Miralles F, Sastre-Garriga J, Tintoré M, et al. Clinical impact of early brain atrophy in clinically isolated syndromes. *Mult Scler Houndmills Basingstoke Engl*. 2013;19(14):1878-1886. doi:10.1177/1352458513488231

29.   Rae-Grant A, Day GS, Marrie RA, et al. Practice guideline recommendations summary: Disease-modifying therapies for adults with multiple sclerosis: Report of the Guideline Development, Dissemination, and Implementation Subcommittee of the American Academy of Neurology. *Neurology*. 2018;90(17):777-788. doi:10.1212/WNL.0000000000005347

30.   Montalban X, Gold R, Thompson AJ, et al. ECTRIMS/EAN Guideline on the pharmacological treatment of people with multiple sclerosis. *Mult Scler Houndmills Basingstoke Engl*. 2018;24(2):96-120. doi:10.1177/1352458517751049

31.   Arrambide G, Iacobaeus E, Amato MP, et al. Aggressive multiple sclerosis (2): Treatment. *Mult Scler J.* 2020;26(9):1045-1063. doi:10.1177/1352458520924595

32.   Thompson AJ, Baranzini SE, Geurts J, Hemmer B, Ciccarelli O. Multiple sclerosis. *Lancet Lond Engl.* 2018;391(10130):1622-1636. doi:10.1016/S0140-6736(18)30481-1

33.   Goldenberg MM. Multiple Sclerosis Review. *Pharm Ther.* 2012;37(3):175-184.

34.   Stoppe M, Busch M, Krizek L, Then Bergh F. Outcome of MS relapses in the era of disease-modifying therapy. *BMC Neurol.* 2017;17(1):151. doi:10.1186/s12883-017-0927-x

35.   Ehler J, Koball S, Sauer M, et al. Response to Therapeutic Plasma Exchange as a Rescue Treatment in Clinically Isolated Syndromes and Acute Worsening of Multiple Sclerosis: A Retrospective Analysis of 90 Patients. *PloS One.* 2015;10(8):e0134583. doi:10.1371/journal.pone.0134583

36.   Dobson R, Giovannoni G. Multiple sclerosis – a review. *Eur J Neurol.* 2019;26(1):27-40. doi:10.1111/ene.13819

37.   Freeman L, Longbrake EE, Coyle PK, Hendin B, Vollmer T. High-Efficacy Therapies for Treatment-Naïve Individuals with Relapsing-Remitting Multiple Sclerosis. *CNS Drugs.* 2022;36(12):1285-1299. doi:10.1007/s40263-022-00965-7

38.   Ontaneda D, Tallantyre EC, Raza PC, et al. Determining the effectiveness of early intensive versus escalation approaches for the treatment of relapsing-remitting multiple sclerosis: The DELIVER-MS study protocol. *Contemp Clin Trials.* 2020;95:106009. doi:10.1016/j.cct.2020.106009

39.   Harding K, Williams O, Willis M, et al. Clinical Outcomes of Escalation vs Early Intensive Disease-Modifying Therapy in Patients With Multiple Sclerosis. *JAMA Neurol.* 2019;76(5):536-541. doi:10.1001/jamaneurol.2018.4905

40.   Häusler D, Häusser-Kinzel S, Feldmann L, et al. Functional characterization of reappearing B cells after anti-CD20 treatment of CNS autoimmune disease. *Proc Natl Acad Sci U S A.* 2018;115(39):9773-9778. doi:10.1073/pnas.1810470115

41.   University of California SFMET, Cree BAC, Hollenbach JA, et al. Silent progression in disease activity–free relapsing multiple sclerosis. *Ann Neurol.* 2019;85(5):653-666. doi:10.1002/ana.25463

42.	Kappos L, Wolinsky JS, Giovannoni G, et al. Contribution of Relapse-Independent Progression vs Relapse-Associated Worsening to Overall Confirmed Disability Accumulation in Typical Relapsing Multiple Sclerosis in a Pooled Analysis of 2 Randomized Clinical Trials. *JAMA Neurol*. 2020;77(9):1132-1140. doi:10.1001/jamaneurol.2020.1568

43.	Giovannoni G, Popescu V, Wuerfel J, et al. Smouldering multiple sclerosis: the 'real MS.' *Ther Adv Neurol Disord*. 2022;15:17562864211066752. doi:10.1177/17562864211066751

44.	Cencioni MT, Genchi A, Brittain G, et al. Immune Reconstitution Following Autologous Hematopoietic Stem Cell Transplantation for Multiple Sclerosis: A Review on Behalf of the EBMT Autoimmune Diseases Working Party. *Front Immunol*. 2022;12:813957. doi:10.3389/fimmu.2021.813957

45.	Openshaw H, Stuve O, Antel JP, et al. Multiple sclerosis flares associated with recombinant granulocyte colony-stimulating factor. *Neurology*. 2000;54(11):2147-2150. doi:10.1212/wnl.54.11.2147

46.	Dubinsky AN, Burt RK, Martin R, Muraro PA. T-cell clones persisting in the circulation after autologous hematopoietic SCT are undetectable in the peripheral CD34+ selected graft. *Bone Marrow Transplant*. 2010;45(2):325-331. doi:10.1038/bmt.2009.139

47.	Sharrack B, Saccardi R, Alexander T, et al. Autologous haematopoietic stem cell transplantation and other cellular therapy in multiple sclerosis and immune-mediated neurological diseases: updated guidelines and recommendations from the EBMT Autoimmune Diseases Working Party (ADWP) and the Joint Accreditation Committee of EBMT and ISCT (JACIE). *Bone Marrow Transplant*. 2020;55(2):283-306. doi:10.1038/s41409-019-0684-0

48.	Mancardi GL, Saccardi R, Filippi M, et al. Autologous hematopoietic stem cell transplantation suppresses Gd-enhanced MRI activity in MS. *Neurology*. 2001;57(1):62-68. doi:10.1212/wnl.57.1.62

49.	Burt RK, Cohen BA, Russell E, et al. Hematopoietic stem cell transplantation for progressive multiple sclerosis: failure of a total body irradiation–based conditioning regimen to prevent disease progression in patients with high disability scores. *Blood*. 2003;102(7):2373-2378. doi:10.1182/blood-2003-03-0877

50.   Nabizadeh F, Pirahesh K, Rafiei N, et al. Autologous Hematopoietic Stem-Cell Transplantation in Multiple Sclerosis: A Systematic Review and Meta-Analysis. *Neurol Ther*. 2022;11(4):1553-1569. doi:10.1007/s40120-022-00389-x

51.   Fassas A, Kimiskidis VK, Sakellari I, et al. Long-term results of stem cell transplantation for MS: a single-center experience. *Neurology*. 2011;76(12):1066-1070. doi:10.1212/WNL.0b013e318211c537

52.   Boffa G, Massacesi L, Inglese M, et al. Long-Term Clinical Outcomes of Hematopoietic Stem Cell Transplantation in Multiple Sclerosis. *Neurology*. Published online January 20, 2021:10.1212/WNL.0000000000011461. doi:10.1212/WNL.0000000000011461

53.   Burt RK, Balabanov R, Burman J, et al. Effect of Nonmyeloablative Hematopoietic Stem Cell Transplantation vs Continued Disease-Modifying Therapy on Disease Progression in Patients With Relapsing-Remitting Multiple Sclerosis: A Randomized Clinical Trial. *JAMA*. 2019;321(2):165-174. doi:10.1001/jama.2018.18743

54.   Signori A, Boffa G, Bovis F, et al. Prevalence of disability improvement as a potential outcome for multiple sclerosis trials. *Mult Scler Houndmills Basingstoke Engl*. 2021;27(5):706-711. doi:10.1177/1352458520936236

55.   Atkins HL, Bowman M, Allan D, et al. Immunoablation and autologous haemopoietic stem-cell transplantation for aggressive multiple sclerosis: a multicentre single-group phase 2 trial. *The Lancet*. 2016;388(10044):576-585. doi:10.1016/S0140-6736(16)30169-6

56.   Roccatagliata L, Rocca M, Valsasina P, et al. The long-term effect of AHSCT on MRI measures of MS evolution: a five-year follow-up study. *Mult Scler Houndmills Basingstoke Engl*. 2007;13(8):1068-1070. doi:10.1177/1352458507076982

57.   Chen JT, Collins DL, Atkins HL, et al. Brain atrophy after immunoablation and stem cell transplantation in multiple sclerosis. *Neurology*. 2006;66(12):1935-1937. doi:10.1212/01.wnl.0000219816.44094.f8

58.   Thebault S, Lee H, Bose G, et al. Neurotoxicity after hematopoietic stem cell transplant in multiple sclerosis. *Ann Clin Transl Neurol*. 2020;7(5):767-775. doi:10.1002/acn3.51045

59.   Lee H, Nakamura K, Narayanan S, et al. Impact of immunoablation and

autologous hematopoietic stem cell transplantation on gray and white matter atrophy in multiple sclerosis. *Mult Scler J.* 2018;24(8):1055-1066. doi:10.1177/1352458517715811

60.   De Stefano N, Arnold DL. Towards a better understanding of pseudoatrophy in the brain of multiple sclerosis patients. *Mult Scler Houndmills Basingstoke Engl.* 2015;21(6):675-676. doi:10.1177/1352458514564494

61.   Lee H, Nakamura K, Narayanan S, et al. Brain volume change after high-dose immunosuppression and autologous hematopoietic cell transplantation for relapsing-remitting multiple sclerosis. *Mult Scler Relat Disord.* 2021;54:103149. doi:10.1016/j.msard.2021.103149

62.   Mancardi G, Sormani MP, Muraro PA, Boffa G, Saccardi R. Intense immunosuppression followed by autologous haematopoietic stem cell transplantation as a therapeutic strategy in aggressive forms of multiple sclerosis. *Mult Scler J.* 2018;24(3):245-255. doi:10.1177/1352458517742532

63.   Willison AG, Ruck T, Lenz G, Hartung HP, Meuth SG. The current standing of autologous haematopoietic stem cell transplantation for the treatment of multiple sclerosis. *J Neurol.* 2022;269(7):3937-3958. doi:10.1007/s00415-022-11063-5

64.   Das J, Snowden JA, Burman J, et al. Autologous haematopoietic stem cell transplantation as a first-line disease-modifying therapy in patients with "aggressive" multiple sclerosis. *Mult Scler Houndmills Basingstoke Engl.* 2021;27(8):1198-1204. doi:10.1177/1352458520985238

65.   Thebault S, Tessier DR, Lee H, et al. High serum neurofilament light chain normalizes after hematopoietic stem cell transplantation for MS. *Neurol - Neuroimmunol Neuroinflammation.* 2019;6(5). doi:10.1212/NXI.0000000000000598

66.   Sormani MP, Muraro PA, Schiavetti I, et al. Autologous hematopoietic stem cell transplantation in multiple sclerosis: A meta-analysis. *Neurology.* 2017;88(22):2115-2122. doi:10.1212/WNL.0000000000003987

67.   Armstrong T, Gilbert MR. Central nervous system toxicity from cancer treatment. *Curr Oncol Rep.* 2004;6(1):11-19. doi:10.1007/s11912-996-0004-x

68.   Soussain C, Ricard D, Fike JR, Mazeron JJ, Psimaras D, Delattre JY. CNS complications of radiotherapy and chemotherapy. *Lancet Lond Engl.* 2009;374(9701):1639-1651. doi:10.1016/S0140-6736(09)61299-X

69.   Cintrón-García J, Guddati AK. Management of CNS toxicity of chemotherapy and targeted agents: 2020. *Am J Cancer Res*. 2020;10(8):2617-2620.

70.   Silberfarb PM. Chemotherapy and Cognitive Defects in Cancer Patients. *Annu Rev Med*. 1983;34(1):35-46. doi:10.1146/annurev.me.34.020183.000343

71.   Loh KP, Janelsins MC, Mohile SG, et al. Chemotherapy-related cognitive impairment in older patients with cancer. *J Geriatr Oncol*. 2016;7(4):270-280. doi:10.1016/j.jgo.2016.04.008

72.   Cognitive impairment associated with adjuvant therapy in breast cancer - PubMed. Accessed April 28, 2023. https://pubmed.ncbi.nlm.nih.gov/16097037/

73.   Wefel JS, Kesler SR, Noll KR, Schagen SB. Clinical characteristics, pathophysiology, and management of noncentral nervous system cancer-related cognitive impairment in adults. *CA Cancer J Clin*. 2015;65(2):123-138. doi:10.3322/caac.21258

74.   Quesnel C, Savard J, Ivers H. Cognitive impairments associated with breast cancer treatments: results from a longitudinal study. *Breast Cancer Res Treat*. 2009;116(1):113-123. doi:10.1007/s10549-008-0114-2

75.   Lv L, Mao S, Dong H, Hu P, Dong R. Pathogenesis, Assessments, and Management of Chemotherapy-Related Cognitive Impairment (CRCI): An Updated Literature Review. *J Oncol*. 2020;2020:e3942439. doi:10.1155/2020/3942439

76.   Deprez S, Amant F, Smeets A, et al. Longitudinal assessment of chemotherapy-induced structural changes in cerebral white matter and its correlation with impaired cognitive functioning. *J Clin Oncol Off J Am Soc Clin Oncol*. 2012;30(3):274-281. doi:10.1200/JCO.2011.36.8571

77.   Abraham J, Haut MW, Moran MT, Filburn S, Lemiuex S, Kuwabara H. Adjuvant chemotherapy for breast cancer: effects on cerebral white matter seen in diffusion tensor imaging. *Clin Breast Cancer*. 2008;8(1):88-91. doi:10.3816/CBC.2008.n.007

78.   Granziera C, Wuerfel J, Barkhof F, et al. Quantitative magnetic resonance imaging towards clinical application in multiple sclerosis. *Brain J Neurol*. 2021;144(5):1296-1311. doi:10.1093/brain/awab029

79.   Dardiotis E, Nousia A, Siokas V, et al. Efficacy of computer-based cognitive training in neuropsychological performance of patients with multiple sclerosis: A

systematic review and meta-analysis. *Mult Scler Relat Disord*. 2018;20:58-66. doi:10.1016/j.msard.2017.12.017

80.   Fragoso YD, Wille PR, Abreu M, et al. Correlation of clinical findings and brain volume data in multiple sclerosis. *J Clin Neurosci Off J Neurosurg Soc Australas*. 2017;44:155-157. doi:10.1016/j.jocn.2017.06.006

81.   Eshaghi A, Prados F, Brownlee WJ, et al. Deep gray matter volume loss drives disability worsening in multiple sclerosis. *Ann Neurol*. 2018;83(2):210-222. doi:10.1002/ana.25145

82.   De Stefano N, Airas L, Grigoriadis N, et al. Clinical relevance of brain volume measures in multiple sclerosis. *CNS Drugs*. 2014;28(2):147-156. doi:10.1007/s40263-014-0140-z

83.   Sormani MP, Arnold DL, De Stefano N. Treatment effect on brain atrophy correlates with treatment effect on disability in multiple sclerosis. *Ann Neurol*. 2014;75(1):43-49. doi:10.1002/ana.24018

84.   De Stefano N, Giorgio A, Gentile G, et al. Dynamics of pseudo-atrophy in RRMS reveals predominant gray matter compartmentalization. *Ann Clin Transl Neurol*. 2021;8(3):623-630. doi:10.1002/acn3.51302

85.   Sastre-Garriga J, Tur C, Pareto D, et al. Brain atrophy in natalizumab-treated patients: A 3-year follow-up. *Mult Scler Houndmills Basingstoke Engl*. 2015;21(6):749-756. doi:10.1177/1352458514556300

86.   Sw S, Hf L, Tq L, Rc A, Ah C, Sk S. Differential sensitivity of in vivo and ex vivo diffusion tensor imaging to evolving optic nerve injury in mice with retinal ischemia. *NeuroImage*. 2006;32(3). doi:10.1016/j.neuroimage.2006.04.212

87.   Kolasa M, Hakulinen U, Brander A, et al. Diffusion tensor imaging and disability progression in multiple sclerosis: A 4-year follow-up study. *Brain Behav*. 2018;9(1):e01194. doi:10.1002/brb3.1194

88.   Lee H, Narayanan S, Brown RA, et al. Brain atrophy after bone marrow transplantation for treatment of multiple sclerosis. *Mult Scler Houndmills Basingstoke Engl*. 2017;23(3):420-431. doi:10.1177/1352458516650992

89.   Inglese M, Mancardi GL, Pagani E, et al. Brain tissue loss occurs after suppression of enhancement in patients with multiple sclerosis treated with autologous haematopoietic stem cell transplantation. *J Neurol Neurosurg Psychiatry*.

2004;75(4):643-644. doi:10.1136/jnnp.2003.021865

90.   Nash RA, Hutton GJ, Racke MK, et al. High-dose immunosuppressive therapy and autologous HCT for relapsing-remitting MS. *Neurology*. 2017;88(9):842-852. doi:10.1212/WNL.0000000000003660

Ringraziamenti

Vorrei cominciare ringraziando innanzitutto la Professoressa Matilde Inglese, la quale mi dato la possibilità di partecipare a questo progetto di ricerca. Ringrazio Giacomo Boffa, che durante il periodo in cui mi ha accompagnato nello svolgimento di questo lavoro ha dimostrato sempre una grandissima passione verso il mondo della ricerca e in tutto quello che fa.

Voglio ringraziare mio padre, la persona che più nella mia vita mi ha insegnato cosa significa dire non solo trovare piacere nel proprio lavoro, ma soprattutto cosa vuol dire innamorarsi della scoperta, dello studio e della ricerca. Sei sempre stato e sempre sarai il mio esempio di ciò si può fare quando si possiede una passione smisurata in ciò per cui si crede e si è pronti a lottare per raggiungerlo. In nessuna persona al mondo ho mai riscontrato una dedizione così inamovibile verso un'idea, un obiettivo, e questa è la cosa che sin da piccolo ho ammirato di più in te. Il mio desiderio in questo momento, all'alba della mia carriera lavorativa, è di ritrovarmi in qualcosa che mi scateni una passione anche solo in parte paragonabile a ciò che provi tu nei confronti di ciò che fai. So che sarai sempre presente per indirizzarmi verso la scelta giusta.

Grazie a mia madre, perché sei sempre stata il mio punto fermo, roccaforte in qualsiasi ambito della vita. Sei stata il mio pilastro emotivo in molte, troppe situazioni. Sei sempre presente in ogni momento di bisogno, subito pronta a mettere da parte qualsiasi cosa per aiutarmi nelle richieste più disparate. Senza di te probabilmente non sarei arrivato, o almeno non tutto intero, fino a questo punto.

A entrambi i miei genitori voglio dire solo una cosa: vi ringrazio della libertà che mi avete sempre concesso, di avermi dato la possibilità in ogni ambito di fare le mie scelte, di avermi sempre dato la possibilità di compiere i miei errori, di sbattere la testa e di correggerli da solo. Vi ringrazio perché, nonostante i problemi, avete sempre messo me e mio fratello davanti ad ogni cosa e ci avete sempre considerato la vostra priorità.

Grazie a Samuele, che in questi sei anni ho visto lentamente maturare da ragazzo a giovane uomo, so che raggiungerai grandi obiettivi nella vita. Hai un potenziale enorme, cosa che non credo tu abbia ancora pienamente realizzato, e spero che te renda conto prossimamente. So che in futuro ci saremo sempre l'uno per l'altro e ci verremo sempre incontro per aiutarci, e questa non è una fortuna che pochi possono dire di

possedere. A Pietro, che quando ho iniziato questo percorso aveva solo 4 anni e adesso è un piccolo ometto, ricordo ancora quando giocavamo insieme a fare il dottore con lo stetoscopio giocattolo. Ricordo ancora quando ti ho fatto da babysitter da solo per la prima volta e non smettevi di piangere, e io, quattordicenne impanicato, non avevo la minima idea di cosa fare.

Ai miei nonni Diana ed Emilio, che, pur non essendo più qui a supportarmi, sono sempre nei miei pensieri.

Ai miei nonni, zii e cugine di Asti, che ringrazio per il supporto costante dimostratomi in tutti questi anni.

Alla compagnia dell'Evergreen, che da sempre è stata la mia seconda famiglia. È raro trovare delle persone che possiedano un senso di gruppo e di unità forte come quello che ritrovo all'interno di questo gruppo. Sempre pronti a fare balotta, sempre pronti a coinvolgermi nelle situazioni più disparate e a tirarmi fuori dal mio piccolo angolino solitario. Ringrazio in particolare Lorenzo, compagno irrinunciabile di infiniti allenamenti, ti sarò sempre debitore per avermi portato in palestra la prima volta. Ringrazio Enrico, che ormai posso dire di conoscere da troppo tempo, e che è sempre stata la persona a instillarmi un po' di buonsenso nelle situazioni critiche, soprattutto durante il liceo, che senza di te sarebbe stata un'esperienza completamente diversa.

Non starò a dilungarmi troppo anche se le cose che devo ai ragazzi dell'Ever sono tante, se non troppe. Ringrazio quindi Francesco, Marco, Giovanni, Piro, Umberto, Guido e tutti gli altri per avere supportato, seppur dalla "lontana" Alessandria, questa mia strada.

DÜMA

Tra gli amici di Alessandria voglio ringraziare anche Federico, il quale ogni volta che tornavo in Alessandria organizzava una partita di padel o mi chiamava per farmi infognare in qualche nuovo gioco online. In ogni caso, devi ancora imparare a battermi a scacchi.

Grazie a Tommaso e a Jacopo, i quali mi hanno sempre accolto in casa loro per le loro cene con partita, soprattutto vi ringrazio per le fantastiche carbonare di Tommaso.

Premetto che questa parte di ringraziamenti sarà la più amara. Amara perché parlerò di persone che ho incontrato, che ho conosciuto, con cui ho forgiato un legame, con cui ho riso, pianto, litigato, vissuto e che hanno dato forma alla mia vita in questi anni. Amara perché, nonostante credo in un sentimento di amicizia eterna tra di noi, so che molte delle quotidianità che siamo spesso abituati a dare per scontato ci verranno rapidamente portate via dall'incessabile scorrere della vita, che per noi, come per tutti gli altri, deve proseguire. Siete le persone in cui oggi ritrovo la maggior parte di me stesso, siete coloro in cui definisco la persona che sono oggi.

Asia e Isabella, vi ringrazio per essere state in questi anni di convivenza le sorelle che non ho mai avuto. Ormai avete imparato a leggermi come un libro, sapete quando sono contento, quando sono depresso, quando cerco di nascondere qualcosa o quando timidamente cerco di confessarvelo. Questo ultimo anno, in particolare, per me sarebbe stato una cosa completamente diversa senza il vostro supporto, i vostri consigli, la vostra voglia di ascoltarmi. Non so in che modo avrei affrontato molte situazioni senza il vostro aiuto. Asia, la tua capacità di sollevarmi il morale in ogni situazione è straordinaria, sei dotata di un'aura radiante che abbraccia tutti quelli che ti stanno attorno donando spensieratezza. Ammiro la tua capacità di dire immediatamente ciò che pensi alle persone, e forse sono un po' invidioso perché in questo siamo diametralmente opposti. Isabella, sei stata la seconda madre, non solo hai garantito la mia sopravvivenza dentro e fuori la casa (i miei prossimi coinquilini ti saranno grati), ma hai sempre avuto la capacità di dispensare il consiglio giusto al momento giusto. Sei dotata di una pazienza infinita, hai sempre grande riguardo in quello che dici per non ferire il prossimo, ma sei anche pronta ad attaccare quando qualcuno esagera.

Un grazie va anche ad Andreina, una persona che non si ferma un attimo, dotata di infinita energia, che ha sempre portato tanta allegria in casa. Ti ringrazio per avermi sopportato quando per ogni esame venivo incessantemente a chiederti consigli, appunti, o venivo solamente a lamentarmi di qualcosa che mi era successo.

E sì, pur non essendo probabilmente sempre stato un coinquilino perfetto (come dimostrano le crocette mancanti nei turni delle pulizie), spero a mio modo di avere portato serenità in quella che ormai era la nostra piccola quotidianità. L'appartamento di Corso Europa occuperà sempre un posto in un angolo del mio cuore.

Grazie a Dafne, la tua capacità di unirci tutti insieme in qualsiasi momento è incomparabile. La tua energia stravolge chi ti sta attorno, è come un fiume che coinvolge e trascina tutte le persone che si ritrovano in mezzo alla corrente. Un fiume in cui ho avuto la fortuna di cadere e che mi ha trascinato verso nuovi, strani, individui che non vedevano l'ora di fare amicizia con un timido ragazzo all'inizio del secondo anno.

Grazie a Pietro, altro cardine fondamentale del gruppo. Grazie perché negli anni hai sempre spinto per festeggiare tutti insieme ogni volta che abbiamo potuto, grazie per le mille cene organizzate in cui mi ha deliziato con la tua cucina.

Grazie ad Alessio per il breve periodo condiviso insieme in casa, sei l'unico nel gruppo con il fisico più grosso del mio e per questo non ti perdonerò mai.

Grazie a Bianca per avere portato un po' di aria di festa e un pizzico di follia in questo ultimo anno (forse anche un po' troppa).

Grazie a Luca per tutte le serate passate con una birra fresca davanti alla playstation, davanti ad una partita, o solamente tra di noi a parlare del più e del meno. Posso solamente dire: "A nastro".

Grazie a Chiara e Camilla, sfortunatamente abbiamo avuto poco tempo per conoscerci pienamente, ma dopo questo breve, folle periodo, tra un ultimo impegnativo esame e una laurea da preparare, mi avete accolto in casa vostra e mi avete fatto sentire come se mi aveste sempre conosciuto. Sono fiducioso sul fatto che in futuro avremo tempo per permettere a questa nuova amicizia di evolvere verso nuovi confini.

Concludo dicendo a tutti voi che le cose per cui dovrei ringraziarvi sono innumerevoli, e probabilmente dilungarmi ancora sarebbe solamente un vano tentativo di esprimere a parole un affetto troppo grande per essere messo nero su bianco. Intanto io, con un groppo alla gola ma infinita fiducia nel legame indistruttibile che abbiamo creato, auguro a tutti voi di riuscire a realizzare pienamente i vostri sogni e di realizzarvi completamente per le persone incredibili che avete sempre dimostrato di essere.

Lasciando tutto questo e avviandomi verso le conclusioni, ringrazio coloro che inevitabilmente non sono potuti essere presenti oggi, ma che mi hanno accompagnato, nonostante tutto, lungo un cammino durato sei anni.

Diverse persone speciali hanno accompagnato questo percorso che si conclude oggi, a tutti voi non posso che dedicare il più grande dei ringraziamenti.

I miei ultimi pensieri vanno a Giovanni, amico e compagno dal primo all'ultimo giorno di questo percorso. Sei stata la persona che più di tutte ha definito la mia vita durante l'Università. Sarebbe quasi banale affermare che è raro incontrare una persona speciale come te, ma sarebbe anche profondamente sbagliato credere che l'esserci trovati immediatamente, quasi come se fosse stato imposto dal destino in quell'ormai lontano primo giorno, non sia stato un evento più unico che raro, probabilmente di quelli che avvengono solamente una volta nell'arco di una vita. Ciò che mi rende felice è sapere che, dopo quel primo giorno, quasi sei anni fa, proprio come abbiamo iniziato insieme la prima lezione, oggi taglieremo questo traguardo non più solamente come compagni di corso, ma come fratelli.

Non dimenticherò mai le risate, i mille meme, le lezioni, le infinite pagine di appunti, tutte le giornate passate insieme a ripetere. Allo stesso modo però non voglio dimenticare neanche la disperazione, l'ansia, le preoccupazioni che hanno contraddistinto questi anni e che abbiamo condiviso insieme. Avere vissuto questo folle percorso insieme, dall'inizio alla fine, è stata un'esperienza meravigliosa, ti ringrazio per avermi accompagnato e avere condiviso con me ogni lato di tutto ciò, migliorando i momenti belli e alleggerendo i momenti brutti.

Mi hai sempre spinto a dare di più e voglio che tu sappia che sei una delle persone che più ammiro per l'impegno che metti nelle cose che fai, caratteristica che ti permette di essere sempre un passo davanti a tutti.

Ti auguro di raggiungere i grandi obiettivi a cui sei destinato, ma sono già sicuro che sarai pienamente in grado di prendere tutto ciò che vuoi dalla vita.

Al nostro prossimo capitolo.