Title: Biomarkers of disease PROgression and myeloid profiling in patients with relapsing remitting multiple sclerosis treated with autologous hematopoietic stem cell TRANSPLANTation and second line therapies.

Protocol: TRANSPLANT-PRO study

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Data: 03/05/2022

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PAGINA DELLE FIRME

Dichiaro che questo protocollo è stato letto attentamente e pienamente compreso. Accetto di seguire le procedure dello studio come descritto in questo protocollo in conformità con la Buona Pratica Clinica e tutti gli altri requisiti normativi.

[redacted] 03/05/2022

Primario U.O. Neurologia per conto del Promotore | **Firma** | **Data**

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[redacted] 03/05/2022

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Summary

1 ABBREVIATION

ADWP	Working Party for Autoimmune Diseases
aHSCT	Autologous hematopoietic stem cell transplantation
AIFA	Italian pharmacological regulatory agency
BBB	Blood Brain Barrier
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
DMTs	Disease modifying treatments
EBMT	European Group for Bone Marrow Transplant
EDSS	Expanded Disability Status Scale
GCIPL	Ganglion cell–inner plexiform layer
HAD	Highly active disease
INL	Inner nuclear layer
MS	Multiple Sclerosis
MTR	magnetization transfer imaging
NADPH	nicotinamide adenine dinucleotide phosphate
NAGM	Normal appearing grey matter
NAWM	Normal appearing white matter
NEDA-3	non evidence of disease activity 3
OCT	Optical coherence tomography
PBMCs	Peripheral blood mononuclear cells
PFS	Progression free survival
PMS	Progressive MS
PPMS	Primary PMS
PRLs	Paramagnetic rim lesions
RNFL	Retinal nerve fiber layer
ROS	Reactive oxygen species
RRMS	Relapsing-remitting MS
SELs	Slowly expanding lesions
sNfL	Serum neurofilament light chain
SPMS	Secondary PMS

2 INTRODUCTION

Multiple sclerosis (MS) physio-pathological mechanisms are characterized by inflammation, demyelination and primary or secondary axonal degeneration[1]. According to the Lublin's new MS phenotypic classification[2], disease course is characterized as relapsing-remitting (RRMS) or progressive MS (PMS), including a consideration of disease activity (evaluated through clinical relapse rate and imaging findings). Primary progressive MS (PPMS) and secondary progressive (SPMS) are considered as part of the PMS spectrum[3]. The pathogenetic mechanisms involved in the disease development are extremely heterogeneous and closely related to the clinical disease phenotypes: RRMS and PMS[4]. Inflammation is a hallmark of MS and a dysregulation of self-tolerance toward myelin and other central nervous system (CNS) antigens induces a persistent peripheral activation of autoreactive cells that become able to cross a leaky blood-brain barrier (BBB). After entering CNS, these cells trigger an inflammatory cascade leading to the release of cytokines and chemokines, recruitment of additional inflammatory cells and persistent activation of microglia and macrophages resulting in myelin damage. Neurodegeneration has a pivotal role in PMS pathophysiological mechanisms, but whether early neurodegeneration drives autoimmune injury, or whether ongoing CNS inflammation reaches a threshold to trigger neurodegeneration, is still unclear[5]. PMS presents, differently from RRMS, a persistent chronic inflammation behind a 'closed' BBB involving primarily the myeloid compartment, and only partially the lymphoid one, with the release of reactive oxygen species (ROS) and nitrogen species contributing to mitochondrial and axonal damage which, at last, leads to neurodegeneration[6]. Indeed, axonal damage is an important contributor to brain atrophy and is associated with increasing disability in patients with PMS[7]. Infiltration of monocytes/macrophages and local activation of microglia and resident macrophages are a pathophysiological hallmark in PMS. Normal appearing white matter (NAWM) and grey matter (NAGM) present diffuse microglial inflammation that correlates with axonal injury[8]. Moreover, myeloid cells seem to drive the slow expansion of chronic active lesions (paramagnetic rim lesions, PRLs) [9]. The clusters of activated microglial cells and macrophages at the edge of the PRLs are important drivers of clinical deterioration, since they exert ongoing tissue damage[10]. Macrophages/microglia present nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity with consequent ROS production and increased neuronal calcium levels, impairing neuronal function. In addition, CNS myeloid cells release proinflammatory cytokines and chemokines, leading to oligodendrocyte and neuronal damage[11]. Iron accumulation in normal NAGM and NAWM could

amplify chronic inflammation, exacerbate mitochondrial dysfunction and potentiate axonal damage[12],[13]. In addition, PRLs and some inactive lesions are surrounded by an iron rim constituted by iron pro-inflammatory macrophages/microglia[9],[14].

Disease modifying therapies (DMTs) approved for MS are highly effective in the relapsing-remitting phase reducing clinical relapses and MRI activity mainly through peripheral immunomodulation.

Since 1996, autologous hematopoietic stem cell transplantation (aHSCT) has been proposed as a treatment in severe cases of MS both in Europe and North America. According to the European Group for Bone Marrow Transplant (EBMT) registry over 1656 patients suffering from MS have received aHSCT. aHSCT preceded by myeloablative conditioning regimens, eradicates inflammatory autoreactive immune clones and restore a new immune tolerance. The use of aHSCT for patients with aggressive RRMS was demonstrated to be highly effective, achieving a long-term clinical remission in most patients. The EBMT Working Party for Autoimmune Diseases (ADWP) recently published guidelines and recommendations for the use of HSCT in MS, recommending as standard of care the transplantation in patients with highly active RRMS failing DMTs[15].

Beside the encouraging role of aHSCT in RRMS in terms of clinical and MRI remission, a few data are available on its role in delaying conversion to SPMS. Studies of the pre-DMTs *era* showed that two-thirds of RRMS patients transit into the debilitating SP phase of the disease within 30 years of diagnosis[16]. Despite DMTs could contribute to a lower risk of higher disability scores and prolong time for conversion to SPMS[17], precise data on progression rates and timing are not yet available. Moreover, among patients with RRMS, initial treatment with highly effective treatments (e.g., fingolimod, alemtuzumab, or natalizumab) was associated with a lower risk of conversion to SPMS compared with lower effective treatments (e.g., glatiramer acetate or interferon beta)[18]. Alemtuzumab (monoclonal antibody direct against the cell surface protein CD52 leading to the depletion of circulating lymphocytes) and ocrelizumab (anti-CD20 monoclonal antibody targeting B cells) are able to achieve a progression free survival (PFS) of 69% at 12 years[19] and 83.9% at 5 years[20], respectively. In patients with highly active disease (HAD) - a population more comparable to the transplanted one - the PFS at 9 years with alemtuzumab drops to 56-64%[21]. In contrast with these DMTs, the main aHSCT ablative conditioning regimen used in Europe (BEAM) depletes both the lymphoid and myeloid pathogenic immune repertoires, with available data showing PFS of 86.8% and 71.3% at 8[22] and 10 years[23], respectively.

The identification of PMS on clinical ground is often retrospective. A challenge in the management of PMS is the identification of sensitive and specific biomarkers capable of identifying progression early and monitoring its evolution. Several imaging markers have been explored such as whole brain atrophy and GM atrophy, along with advanced MRI measures including magnetization transfer imaging (MTR). Cortical lesion burden and accumulation over time correlate with disability and cognitive impairment[24]. In a 7-year follow-up study in RRMS patients, a higher number of cortical lesions (≥7) at disease onset predicted conversion to SPMS after a shorter period of time[25]. PRLs represent another promising MRI biomarker of progression. After the acute phase of inflammatory demyelination, a newly formed enhancing lesion can display different pathological and repair outcomes and can become: 1) chronic active with a PRLs, 2) chronic inactive without a paramagnetic rim, and 3) partially or completely repaired (remyelinated). Interestingly the lesion fate is dictated approximately within the first 3 months after the acute phase[26]. Among different pathological lesion types, PRLs are of special clinical and biological interest for their accumulation of iron-rich activated microglia and/or macrophages at the lesion edge, subtle opening of the BBB, and repair/remyelination failure with axonal loss. In PRLs the general tendency of lesions to collapse due to prominent axonal loss is counterbalanced by ongoing inflammatory demyelination at the lesion edge. PRLs can be identified on 7-T and 3-T susceptibility-based MRI in vivo as non-gadolinium-enhancing chronic lesions with a paramagnetic rim[27,28]. Most patients with MS (56%) had at least 1 PRLs independently of clinical phenotype and DMT. Patients with higher number of PRLs have more aggressive disease (higher lesion load, larger ventricular volumes and lower white matter and basal ganglia volumes), reach higher motor and cognitive disability and transit to disease progression at younger age, despite treatment. PRLs neuropathology evaluations demonstrated the presence of a destructive process at core (pronounced axonal los) and edge (smoldering inflammation and demyelination affecting surrounding tissue). Elliott et al. evaluated the proportion of slowly expanding lesions (SELs) on conventional T1- and T2-weighted sequences in patients with PPMS treated with ocrelizumab compared to placebo, showing in the treated patients a lower proportion of total pre-existing T2-hyperintense lesions identified as SEL, a lower T1- hypointense lesion volume increase in SELs and a significant reduced decrease in normalized T1 signal intensity in SELs[29]. Nevertheless, this study did not evaluate the presence of a paramagnetic rim, that reflect the presence of activated microglia at the lesions edge. Indeed, the SELs number does not fully reflect the presence of PRLs and their pathogenetic role. In a poster presented at ECTRIMS congress 2021, Ben Ayad et al. demonstrated

that patients treated with ocrelizumab did not show a clear treatment effect on PRL volume, as most PRL remained stable or expanded over time in treated patients and controls. These data support the notion that microglia/macrophage–mediated inflammation is a prominent feature of MS that is not the primary target of current treatments.

Other potential biomarkers of progression include, retinal nerve fiber layer (RNFL) at optical coherence tomography (OCT) and serum neurofilament light chain (sNfL). OCT in MS has been widely used to measure in particular RNFL and ganglion cell–inner plexiform layer (GCIPL) thickness as markers of neuroaxonal loss, allowing to detect subclinical neurodegeneration, independently predicting long-term disability in RRMS patients[30]. More recently, inner nuclear layer (INL) - another retinal layers - received attention as possible biomarkers in MS. Pathology studies have identified inflammation and microglial activation within the inner retina in MS patients[31], and in vivo observations also suggest INL as a possible biomarker of inflammation within the CNS, with increased INL thickness reflecting a condition of retinal inflammation, which parallels brain inflammatory activity in MS: microcystic macular edema within this layer has infact been described to be associated with disability. sNfL is a plausible marker of neurodegeneration that can be measured accurately, sensitively, and reproducibly. sNfL correlates with imaging and disability measures, predict the future course of the disease, and can predict response to treatment[32]. Chronic white matter inflammation is associated with increased levels of sNfL and disease severity in MS patients without acute disease activity, suggesting that PRL contribute to clinically relevant, inflammation-driven neurodegeneration[33].

3 RATIONALE AND SPECIFIC AIMS

Due to the limited availability of treatment in PMS, an in dept analysis to better understand (1) the effect of DMTs in preventing transition to SPMS and (2) the progression-related pathogenetic mechanisms, is essential. This could contribute to change the MS therapeutic perspective halting the progression independent of relapse activity putative processes, beside the prevention of relapse associated worsening. Myeloablative aHSCT in RRMS, differently from other widely used highly effective DMTs such as ocrelizumab and alemtuzumab, could modulate the myeloid activity inducing - after the depletion induced by conditioning regimen - a homeostatic expansion and enhanced immune regulation of monocytes/macrophages and dendritic cells. Currently used DMTs do not primary target microglia/macrophage–mediated inflammation, and the effect on the abovementioned

immune population could account for the advantage of aHSCT, compared to ocrelizumab and alemtuzumab, on PFS. Indeed, alemtuzumab and ocrelizumab achieve a long-term PFS lower than aHSCT. The results of such analyses could guide clinical decisions that will have a long-term impact, given the chronicity of the diseases, the duration of therapies, and the long-lasting effects of some treatments **(Figure 1).**

Given this premise, by evaluating n.10 consecutively recruited patients with RRMS treated with myeloablative aHSCT in comparison with patients (n.10 per group) treated with anti-CD52 monoclonal antibody (alemtuzumab) and anti-CD20 monoclonal antibody (ocrelizumab or ofatumumab), the aims of this longitudinal study are the following:

3.1 Primary Aim

Aim 1: To evaluate impact of the studied treatments (myeloablative aHSCT, alemtuzumab and ocrelizumab/ofatumumab) on biomarkers of disease progression in MS. Since to clinically evaluate conversion to SPMS a long follow up is required, the evaluation of progression's surrogate biomarkers (clinical, neuroradiological and biological) will allow a better and faster identification of the disease course.

3.2 Secondary Aims

Aim 2: To characterize the myeloid compartments' longitudinal changes induced by each treatment (aHSCT, alemtuzumab and ocrelizumab/ofatumumab) in the enrolled patients. The blood immune profile will be analyzed over time. In selected patients, selected according to hematologist indications, myeloid progenitors and the hematopoietic niche at bone marrow level will be studied.

Aim3: To explore a correlation between characteristics of the myeloid profile and surrogate endpoints of disease progression, assessing whether the treatment-induced homeostatic expansion and enhanced immune regulation of the myeloid compartment are related to surrogate endpoints of progression.

3.3 Primary endpoint

Evolution of the paramagnetic rim lesions (PRLs), main biomarker of progression, evaluated longitudinally at baseline and at 6, 12 and 24 months after study treatment (see section 5 "Analysis 1").

3.4 Secondary endpoints

- Other biomarkers of progression (multiple sclerosis functional composite score (MSFC), serum neurofilament light chain (sNfL), retinal nerve fiber layer (RNFL), cortical lesions, cerebral atrophy (GM, WM)) evaluated longitudinally (for details see section 5 "Analysis 1");
- Peripheral blood myeloid line subpopulations changes induced by each therapy studied by cytofluorometric analysis at baseline and at 3, 6, 12 and 24 months after study treatment (see section 5 "Analysis 2");
- Correlation between changes in myeloid subpopulations and progression biomarkers (see section 5 "Analysis 3");

Furthermore, we will evaluate the following treatment related parameters:

- Relapse-free survival
- MRI inflammatory activity–free survival
- Progression-free survival,
- NEDA-3 status (non evidence of disease activity 3)
- Time to first relapse
- Time to first MRI inflammatory activity
- Treatment-free survival for patients treated with myeloablative aHSCT or alemtuzumab
- Early adverse events (occurring <100 days after treatment)
- Late adverse events (occurring >100 days after treatment)
- Changes in quality-of-life measures

4 STUDY DESIGN

The present study is a monocentric, prospective pharmacological, observational study with additional collection of biological material **(Figure 2)**.

The study is observational and is not designed to modify the clinical history of the disease.

The decision to prescribe treatment, according to current clinical practice and guidelines, is entirely independent of the decision to include the patient in the study. Furthermore, the therapeutic choice of one drug over another will be dictated by clinical indications and not by the study itself.

The study is configured as monocentric, as the San Raffaele Hospital, through the Multiple Sclerosis Center of the Neurology Unit, is able to collect the planned number of patients.

4.1 Study duration

The study foresees an enrollment phase of 36 months. A total study duration (including enrollment, patient clinical follow-up, biological sample testing, and data analysis) is expected to be 60 months.

4.2 Study population

A total of 30 male or female prospective patients with RRMS who, from clinical practice, may be referred for treatment with aHSCT, alemtuzumab or ocrelizumab/ofatumumab will be recruited. Treatment choice will be independent of participation in the study and should not be initiated with the aim of including the patient in the study. All patients will be required to sign an informed consent prior to the collection of data and biological material.

4.2.1 Eligibility criteria

Inclusion criteria

- Age ≥18 years;
- Signed written informed consent;
- A diagnosis of RRMS according to the 2017 Revisions of the McDonald Criteria;
- High clinical and MRI inflammatory disease activity (at least 2 clinical relapses, or one clinical relapse with Gd- enhancing or new T2 MRI lesions at a separate time point, in the previous 12 months)
- Patients referred for pharmacological treatment with aHSCT, alemtuzumab or ocrelizumab /ofatumumab, according clinical practice following the Italian pharmacological regulatory agency (AIFA) criteria and guidelines and recommendations from the European Society for Blood and Marrow Transplantation (EBMT) Autoimmune Diseases Working Party (ADWP) and the Joint Accreditation Committee of EBMT and ISCT (JACIE);

Exclusion criteria

- Diagnosis of PPMS or SPMS according to the 2017 McDonald criteria
- Known intolerances/allergies to the active substance or to the excipients contained in the DMT and/or contraindications according to product information

The enrolled subjects will be divided into the following groups:

- **Group 1:** patients with RRMS patients who have been prescribed treatment with aHSCT (n.10) as standard indication according to guidelines and recommendations from the European Society for Blood and Marrow Transplantation (EBMT) Autoimmune Diseases Working Party (ADWP) and the Joint Accreditation Committee of EBMT and ISCT (JACIE);
- **Group 2:** patients with RRMS who have been prescribed treatment with ocrelizumab or ofatumumab (n.10) according to Italian pharmacological regulatory agency (AIFA) criteria.
- **Group 3:** patients with RRMS who have been prescribed treatment with alemtuzumab (n.10) according to Italian pharmacological regulatory agency (AIFA) criteria.

5 STUDY PROCEDURES

This study plans the longitudinal collection of demographics, clinical, neurophysiological, neuroradiological, biological and laboratory characteristics of the enrolled patients.

Enrollment visit: baseline evaluation to assess the patients' eligibility (inclusion/exclusion criteria must be satisfied). Patients will be explained all the purposes and details of the study, and will be asked to sign the informed consent for the study and for the biological bank of the IRCCS San Raffaele Hospital (Centro risorse biologiche, CRB).
A clinical, neurophysiological and neuroradiological follow-up will be performed according clinical practice. Usually, clinical evaluation is performed every 3 months after the treatment initiation for the three groups of patients.

Serial collection of peripheral venous blood: Patients will be required to undergo peripheral venous blood sampling at precise timings after the treatment initiation. In particular, in order to study the kinetics of cellular products and plasma concentration of surrogate biomarkers candidates to evaluate disease progression, serial samples will be performed. To minimize discomfort for the patient, the blood sample will be performed simultaneously with the execution of routine visits during the stay in

our department. In particular, to assess treatment-related changes in myeloid subpopulations (e.g. monocytes, dendritic cells) and blood biomarkers (e.g. sNfL), blood samples will be obtained at baseline and at 3, 6, 12 and 24 months from the treatment initiation.

Since aHSCT represents a complex and articulated procedure, in this group of patients, we will evaluate also a blood sample obtained after the stem cells mobilization (collected the same day of stem cells apheresis) and, when available, advanced material from autologous graft infusion. Interestingly, these time points could reflect the bone marrow's microenvironment characteristics.

In order to maintain the pseudo-anonymity of the recruited patients, each test tube will be marked with a progressive number, corresponding to an individual, whose details will be stored in an encrypted database accessible only to the medical staff involved in the study. At baseline demographic and clinical data will be collected.

The project will be organized in three main analyses.

Analysis 1: Identify and evaluate surrogate endpoints of progression in patients with RRMS treated with aHSCT, alemtuzumab and ocrelizumab/ofatumumab. On clinical ground the identification of PMS is often retrospective and a prospective evaluation need very long follow up. A challenge in the management of PMS is the identification of sensitive and specific biomarkers capable of identifying progression early and monitoring its evolution.

The primary surrogate endpoint of progression is represented by paramagnetic rim lesions (PRLs). We will longitudinally evaluate the proportion of stable vs. fading/disappearing PRLs in each group of patients assessing. Paramagnetic rim in these lesions reflect perilesional chronic inflammation and, in particular, iron-laden microglia/macrophage accumulation at the lesion edge. PRLs will be evaluated on MRI using susceptibility-weighted acquisitions.

A chronic lesion will be defined as a PRL when it showed a hypointense rim on phase images, as well as an isointense central area relative to NAWM. MRI images will be acquired for clinical use, including postcontrast T1-weighted sequences, at baseline, 6, 12 and 24 months after the treatments.

Beside PRLs, we will perform a multimodal assessment of surrogate endpoints of progression:

1. Clinical Biomarkers

A comprehensive neurological examination will be longitudinally performed as clinical practice (at baseline, at 3, 6, 12 and 24 months from the treatment initiation) to assess (i) the overall neurological status and longitudinal changes in EDSS score and its functional system sub-scores, Timed 25-Foot Walk (T25FW), 9-Hole Peg Test (9-HPT), symbol digit modality test (SDMT), and multiple sclerosis functional composite score (MSFC); (ii) proportion of disability progression free survival at the end of follow up. Sustained disability progression is defined as an increase of ≥1.5, 1.0 or 0.5 EDSS points, confirmed after a 3- and 6-month relapse-free period, when baseline EDSS score was 0, ≤5.5 or ≥6.0, respectively. This change has not to be attributable to another etiology (i.e. fever, concurrent illness, MS relapse or concomitant medication); (iii) relapse defines as any perceived change in symptoms or signs that persist for ≥ 48 hours in the absence of flu or fever and that is accompanied by objective changes on EDSS functional subscores (FSS) that leads to either a 1.0 point change in EDSS (for baseline EDSS 3 - 5.0) or a 0.5 change (for baseline EDSS≥5.5) together with FSS changes of 2 points in any one FSS or 1 point in any 2 FS. Only relapses meeting the protocol definition will be considered as measure of efficacy.

2. Neurophysiological biomarkers

Longitudinal evaluation of RNFL and GCIPL thickness by OCT will be performed at baseline, 12 and 24 months after the treatment in each group of patients as clinical practice. RNFL and GCIPL are markers of neuroaxonal loss, allowing to detect subclinical neurodegeneration, and to independently predict long-term disability in RRMS patients.

3. Biological biomarkers

We will evaluate serum neurofilament light chain (sNfL) and chitinase 3-like 1 (CHI3L1) levels evaluated at baseline, 6, 12, and 24 months after the transplantation. sNfL is a plausible marker of neurodegeneration that can be measured accurately, sensitively, and reproducibly. sNfL correlates with imaging and disability measures, predict the future course of the disease, and can predict response to treatment. CHI3L1 in MS patients is associated with the development of neurological disability in different clinical phases of the disease.

4. MRI biomarkers

We will longitudinally evaluate (at baseline, 6, 12 and 24 months after the treatments):

- the number and extension of cortical lesions using a double inversion recovery (DIR) sequence. Cortical lesions burden and accumulation over time are higher in PMS, and correlate with disability and cognitive impairment.
- atrophy the whole brain, GM, and WM. Quantification of tissue volume loss (atrophy) from MRI scans is commonly accepted as an in vivo measure of neurodegeneration and helps to predict disease progression.
- lesion, NAWM and GM magnetization transfer ratio (MTR). Magnetization transfer imaging studies have consistently detected more widespread and severe NAWM microstructural tissue abnormalities in PMS patients compared to the other clinical phenotypes, representing a marker of disability progression and cognitive disfunction. MTR measures correlate with axonal degeneration and microglia activation.

Analysis 2: To understand how immunosuppressive treatments in patients with RRMS (aHSCT, alemtuzumab and ocrelizumab/ofatumumab) can modulate the myeloid peripheral landscape. The role of myeloid cells in neuroinflammation and MS pathogenesis calls our attention to the use of different therapeutic agents that target these cells. If only few mechanistic studies on the pathogenetic role of myeloid lineage cells in MS are available, there is even less evidences on the effect of various DMTs on these cells. Notably, we hypothesized that aHSCT could have an advantage, compared to alemtuzumab and ocrelizumab/ofatumumab on disease progression (evaluated through surrogate endpoints) given its ability to suppress the myeloid compartment and stimulate, after HSCs transplantation, a homeostatic expansion - in a microenvironment not inflammatory-prone - of monocytes/macrophages and dendritic cells subpopulations. In selected patients treated with aHSCT, according to hematologist indications, myeloid progenitors and the hematopoietic niche at bone marrow level will be studied (samples collected during apheresis).

For this purpose, we aim to longitudinally analyze the peripheral myeloid subpopulations in each group of treated patients (aHSCT, alemtuzumab and ocrelizumab/ofatumumab) through a flow cytometric analysis on peripheral blood samples obtained at baseline, 3, 6 and 12 and 24 months after the treatment, to evaluate impact of each therapy analyzing the surface markers of several lineage and identifying treatment-related changes in the myeloid lineage. Additionally, in patients treated with aHSCT we will study blood samples obtained after the stem cells mobilization (collected the same day of stem cells apheresis) and, when available, advanced material from autologous graft infusion.

Since immune cells activity is strictly interconnected and the lymphoid and myeloid compartments interact by regulating each other's activity, results of the lymphoid subsets evaluation through immunophenotyping performed according to clinical practice will be studied.

Analysis 3: The last part of the project will try to evaluate whether treatment-related changes in peripheral myeloid signature could condition the pathogenic mechanisms of disease progression. Indeed, in the last decade advancements in technologies such as single-cell RNA sequencing and lineage tracing have highlighted the contribution of monocyte-derived cells enter the CNS parenchyma from the periphery, which becomes crucial during pathological conditions. These evidences demonstrated that CNS myeloid landscape is not a static system embryonically determined, but blood-borne monocytes-derived macrophages and dendritic cells could contribute to neurodegenerative pathogenetic mechanisms beside resident microglia and border associated macrophages (BAMs), often developing a phenotype indistinguishable from resident myeloid cells through classical markers. Given these evidences we hypothesized that DMTs acting on peripheral myeloid cells during the relapsing remitting phase of the disease could condition and regulate CNS compartmentalized neurodegenerative processes related to disability progression. For this purpose, we aim to evaluate whether treatment-related changes in myeloid signature correlate with the above-mentioned surrogate biomarkers of progression, focusing on PRLs. Indeed, at the PRLs edge the microglia/macrophage accumulation plays a pivotal role, mediated by complement component 1q (C1q), in neurodegeneration; these lesions correlate with accumulation of disability and elevated levels of sNfL, representing a biomarker of active axonal injury and transection at the inflammatory lesion edge.

As regarding the follow up, the visit will be scheduled according clinical practice. Clinical visits are usually performed 1 month after a DMTs initiation, and then every 3 months from the treatment initiation. The first MRI and OCT are usually performed 6 months after the treatment initiation or according to clinical/patient needs, the following MRI and OCT are usually performed annually or according to patient's needs. A database will be created with the collected demographic, clinical and instrumental information obtained in the context of normal diagnostic procedures in patients with RRMS.

The study will be end when the last enrolled patients will conclude the 2-year follow up.

5.1 Flowchart

	Enrollment visit	Visit at 3 months after treatment	Visit at 6 months after treatment	Visit at 12 months after treatment	Visit at 24 months after treatment
Informed consent	x				
Treatments: - aHSCT - ALEM - OCR/OFA	x				
Blood samples collection §	x	x	x	x	x
Additional volume of blood samples after mobilization and, when available, from graft infusion * §	x				
Myeloid peripheral landscape/subpopulations analyses	x	x	x	x	x
Biological biomarkers (sNfL, CHI3L1) analyses	x		x	x	x
Clinical biomarkers	x	x	x	x	x
Neurophysiological biomarkers (RNFL, GCIPL)	x			x	x
MRI biomarkers (including Paramagnetic Rim Lesions (PRLs))	x		x	x	x

§ Additional procedure
* Only for patients treated with aHSCT

6 STUDY VARIABLES

Please refer to the attached document (CRF TRANSPLANT-PRO study) for a detailed lists of the variables collected in the study.

7 BIOLOGICAL SAMPLES MANAGEMENT

We will collect the following sample from enrolled patients with RRMS:

- Peripheral blood (about 30 ml) in Ethylenediamine tetraacetic acid (EDTA)

The above-mentioned sample will be collected at baseline and at 3, 6, 12 and 24 months after the treatment's initiation.

In patients treated with aHSCT we will collect also:

- a blood sample obtained after the stem cells mobilization (collected the same day of stem cells apheresis) and, when available, advanced material from autologous graft infusion.

Plasma will be obtained by centrifugation and stored in aliquots at -80°C. Peripheral blood mononuclear cells (PBMCs) will be isolated by Ficoll gradient purification, counted, and frozen in 10% Dimethyl sulfoxide (DMSO). A whole blood sample will be frozen in 10% DMSO.

The samples will be analyzed at the Neuroimmunology unit and the unused material will be stored for almost ten years at the CRB Biobank, for related future studies, e.g. determinations of cellular products that emerge as potential objects of analysis and genomic analysis on DNA or RNA. Samples will be destroyed at the end of the 10 years' time of storage.

Plasma samples will also be analyzed for sNfL content using the The Simoa® Human Neurology 4-Plex A (N4PA) digital immunoassay on an HD-1 Single molecule array (Simoa) instrument according to instructions from the manufacturer (Quanterix, Lexington, MA, USA). PBMCs and whole blood samples will be stained and then acquired on FACS Canto II o FACS Symphony (BD Biosciences) and analyzed with FlowJo software (Tree Star).

8 PATIENT SAFETY AND TREATMENTS AND DIAGNOSTIC WORK-UP

The treatments to which patients will undergo are chosen for clinical reasons independent of this study and are therefore performed for clinical practice according to the indications of use reported in the RCPs approved by EMA and AIFA (attached). There will be no treatments and / or diagnostic paths outside of clinical practice.

Drugs currently approved for the treatment of RRMS patients with high clinical and MRI inflammatory disease activity (at least 2 clinical relapses, or one clinical relapse with Gd- enhancing or new T2 MRI lesions at a separate time point, in the previous 12 months) are natalizumab, ocrelizumab, ofatumumab, alemtuzumab and aHSCT. For the present study we decided to evaluate immunosuppressive treatments (ocrelizumab, ofatumumab, alemtuzumab and aHSCT) and excluded natalizumab due to the different mechanism of action (immunosequestrant vs. immunosuppressive).

9 SIDE EFFECTS AND MANAGEMENT OF ADVERSE REACTIONS

Adverse events will be managed according to the post-marketing legislation: it is responsibility of the promoter/investigator to notify (as normal clinical practice) any adverse reaction occurring during the study according to the norm issued by AIFA on 20/03/2008, which applies to any adverse reaction observed by doctors or other healthcare professional, on the basis of the Decreto Ministeriale del DM issued on 30/04/2015 and following updates.

In parallel, the investigator will also notify the person in charge of the pharmacovigilance of Ospedale San Raffaele (the Director of the hospital pharmacy), by completing the adverse reaction form within two days from being aware of the reaction or within 36 hours if the reaction follows the administration of biological drugs, including vaccines.

It should also be noted that the management of adverse events and reactions related to the drugs (ocrelizumab, alemtuzumab) and procedures used has already taken place for those retrospective patience in accordance with the rules in force for spontaneous reporting (post-marketing).

9.1 Definitions

Adverse Event (AE)	Any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An adverse event (AE) can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product. Reference: GCP ICH E6(R2)
Adverse Drug Reaction (ADR)	In the pre-approval clinical experience with a new medicinal product or its new usages, particularly as the therapeutic dose(s) may not be established: all noxious and unintended responses to a medicinal product related to any dose should be considered adverse drug reactions. The phrase responses to a medicinal product means that a causal relationship between a medicinal product and an adverse event is at least a reasonable possibility, i.e., the relationship cannot be ruled out. Regarding marketed medicinal products: a response to a drug which is noxious and unintended, and which occurs at doses normally used in man for prophylaxis, diagnosis, or therapy of diseases or for modification of physiological function. Reference: GCP ICH E6(R2)
Serious Adverse Event (SAE) or Serious Adverse Drug Reaction (Serious ADR)	Any untoward medical occurrence that at any dose: – results in death, – is life-threatening, – requires inpatient hospitalization or prolongation of existing hospitalization, – results in persistent or significant disability/incapacity, or – is a congenital anomaly/birth defect. Reference: GCP ICH E6(R2)

Suspected Unexpected Serious Adverse Reaction (SUSAR)	An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended. ''Unexpected,'' as used in this definition, also refers to adverse events or suspected adverse reactions that are mentioned in the investigator brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug but are not specifically mentioned as occurring with the particular drug under investigation.

10 STATISTICAL CONSIDERATION

Since main aim consists in evaluation of longitudinal evaluation of neuroradiological and biological surrogate biomarkers of progression in MS in each treatment groups, statistical analysis will be based on longitudinal models. Indeed, data will be analyzed longitudinally, in order to account for individual patterns of change over time. Linear Mixed-Effects (LME) models and Non-Linear Mixed-Effects (NLME) models will be used depending on the shape of the time trend. These are an extension of classic statistical procedures that provides flexibility in the analysis of longitudinal data and are feasible also with small sample sizes.

They allow to model the covariance structure of the data and to characterize individual behavior through random effects. This class of models represent an appropriate methodology for analyzing longitudinal data, accounting for the heterogeneity of the subjects as typically shown in MS data. In LME models, patient unobservable heterogeneity can be accounted on baseline (random effect on the intercept) and/or over time (random effect on the slope): this are used whenever the plot of the data suggests a linear trend. In NLME models, the heterogeneity is accounted by setting random effects on specific parameters of the model that define different characteristics of the nonlinear trajectory. Several forms of non-linearity can be considered depending on either the biological constraint or the data trend. In general, whenever measures of "estimation reliability and robustness" are required, we rely on the standard error of the estimate of the single parameters and on p-values which measure in probability evidence provided by the data under the null hypothesis, that is the probability of no impact for each covariate. We will consider alpha = 0.05 as threshold.

11 DATA MANAGEMENT

The data must be promptly entered in the data collection forms in paper format (attached). The list of information collected must be signed and dated by the principal investigator or by a member of the

designated research team. All data collection sheets and all source documents (i.e. laboratory reports, medical history, physical and diagnostic test results, diagnosis and procedure data, laboratory test results) that support each subject's data collection sheets will be kept in the archives of the investigator responsible for at least ten years after the trial is completed. In the event that the responsible investigator withdraws, relocates or for other reasons relieves himself of the responsibility of archiving the recordings of the study, the custody of the (sealed) recordings must be transferred to another person who accepts this responsibility. All the collected data will be reported in an encrypted database.

12 DATA CONFIDENTIALITY

During the trial, a unique identification code will be assigned to each of the enrolled patients in order to encrypt any possibility of recognition and all data will be processed in a coded manner. The data that will be collected during the study, with the exception of the patient's name, will be recorded, processed and stored together with this code, the patient's date of birth, sex, weight, height and all clinical data relating to his state of health. Only the doctor and authorized subjects will be able to link this code to the patient's name.

To ensure confidentiality, the tubes containing biological samples will also be labeled with specific codes relating to the one attributed to the patient. The data will therefore be processed in ways that allow the identity of the subject from whom biological material is taken for the execution of the analyzes to be uniquely ascertained, but which allow the identification of patients only for the time necessary through the use of a code. Therefore, every appropriate method will be adopted to allow the data subject to be identified only in case of need, separating the identification data where possible. The biological samples will be stored, transported and used in ways aimed also at guaranteeing their quality, integrity, availability and traceability.

The data indicated above will be collected, managed and stored, both in paper and electronic format and in any case processed in compliance with the legislation on the processing of personal data, including the applicable provisions and authorizations issued by the Guarantor Authority for the Protection of Personal Data.

At the end of this process and in the continuation of the study, patient data will be treated completely anonymously.

The patient-related samples will be stored in the CRB Biobank of the San Raffaele Hospital.

13 ETHICAL ASPECTS AND INFORMED CONSENT

This project will be conducted in accordance with all local and national regulations and in accordance with the principles of the World Medical Assembly in Helsinki (1964) and the CH guidelines for the GCP. All patients will be adequately informed and will be asked to sign an informed consent (IC), after discussion and sharing of any doubts with the specialist medical staff. The IC will be drawn up in Italian and the Ethics Committee of our institute will verify its consistency with what is described in the Protocol. This study will be initiated after approval by the Ethics Committee of the San Raffaele hospital in Milan. The informed consent form must state, among other provisions, that the patient is able to understand and consequently accept the conditions of the study as indicated below, in particular he agrees to donate the biological samples provided. Each subject must sign and date an updated informed consent form upon receipt of a detailed explanation of the nature of the study. The subjects will be recruited on a voluntary basis and after signing the informed consent, which also includes the request for authorization to use personal data. As reported above, this protocol does not present physical, psychological and social risks in the procedures that will be followed in the research. If an amendment to the protocol results in a modification of the IC form, a new IC form will be submitted for the attention of the medical Ethics Committee of the study site. It is the investigator's responsibility to ensure that each member of the medical team has received adequate training in accordance with the aforementioned instructions and to keep accurate records. The investigator selected for this clinical program will be responsible for observing the protocol and providing the data relating to the procedure. The investigator must also: accept the requirements of this protocol by placing his / her signature on the protocol signature page; obtain the patient's informed consent in accordance with local regulations; provide the data requested from the information list to be collected as soon as they are available.

Informed consent: The submission of informed consent and the contextual enrollment of patients are subject to the approval of the protocol by the Ethics Committee of the San Raffaele Hospital. Informed consent and the proposal for enrollment for the study will be proposed to patients with relapsing-remitting multiple sclerosis. Patients with relapsing-remitting multiple sclerosis will be recruited through the collaboration of the head of the Neurology Unit. Informed consent involves the donation of biological samples such as blood sampling. Participation in the study does not in itself imply risks. The informed consent provided includes a section relating to the authorization of personal

data for research purposes, and the approval of the study by the Ethics Committee of the San Raffaele Hospital.

14 ECONOMIC AND INSURANCE ASPECTS

This study is of a non-profit nature. The costs of collection, storage at CRB and analyzes on biological samples will be covered by the funding 10A2401 (Professor Filippi).

Given the observational nature of the study, no *ad hoc* insurance coverage is required. Any damage from additional procedures will be covered by OSR.

15 PROPERTY OF DATA AND METHOD OF PUBBLICATION

The data and results generated by the study are owned by IRCCS San Raffaele Hospital.

The results of the study will be made public anonymously through conference presentations and scientific publications. In no case is the publication of data that allow the patient to be recognized.

The results of this study will be submitted to national and international journals; posters and oral communications will be submitted to national and international congresses. All publications and communications regarding the study will be approved in advance by the PI.

The study sponsor / Principal Investigator is responsible for preparing an annual clinical study report to be sent to the Ethics Committee and preparing a final clinical study report. After the data is fully analyzed, it will be communicated, anonymously, to all researchers involved in the study.

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17 FIGURES

Figure 1. Study hypotesis

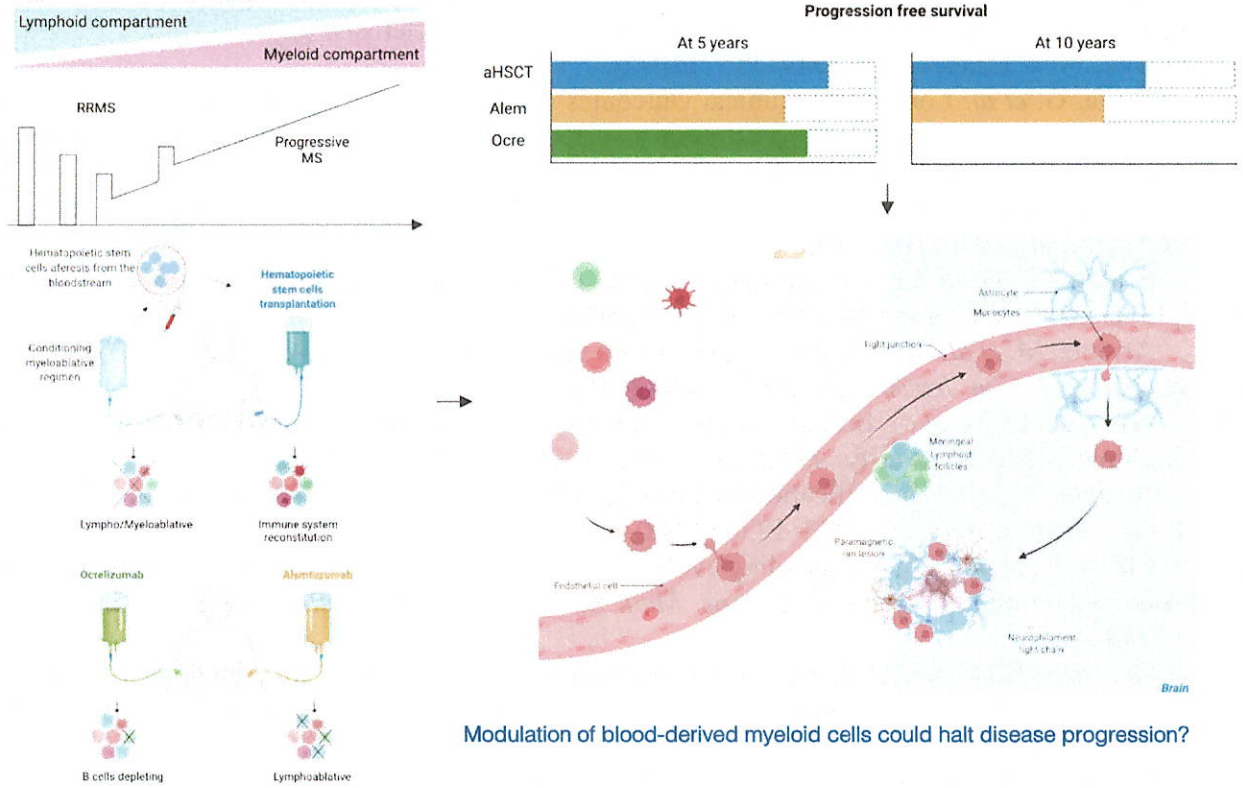

Figure 2: Study design