Study Protocol and Statistical Analysis Plan

Title: Open-label, Observational, Prospective, 9-month Study to Assess the Efficacy of Ofatumumab on Microglia in Patients with Relapsing Forms of Multiple Sclerosis

NCT number: NCT04510220

Date: December 28, 2023

Background and Significance

Historical background

Role of microglia in MS:

Multiple sclerosis (MS) is primarily a demyelinating disease of the central nervous system (CNS), but many patients also undergo progressive atrophy, especially in the gray matter (GM). GM atrophy plays a particularly prominent role in cognitive and physical decline in MS. White matter (WM) demyelination is also correlated with cognitive and physical impairment, albeit GM pathology accounts for the most variance in predicting cognitive and physical impairment.

Both neuroinflammation and neurodegeneration occur during all stages of MS.[1] Axonal loss underlies especially the transition from relapsing to progressive MS, but neurodegeneration with axonal transections is known to be present already early in the disease. By studying progressive MS, one may thus gain important insights about the degenerative component of MS present in all stages of the disease.

Microglia are myeloid cells of the CNS are derived from early embryonic erythromyeloid progenitor cells and have migrated into the developing nervous tissue, where they have begun the maturation process into brain-resident microglial cells. [2] Microglia constitute approximately 10% of the total cell population in a human brain. The key tasks performed by microglia include phagocytosis of extracellular debris, antigen presentation to host immune cells, and secretion of inflammatory mediators. [3]

In the early phase of MS, microglial activation promotes the recruitment of naïve T cells, which are activated in the periphery by dendritic cells functioning as antigen-presenting cells. Microglia also act as antigen-presenting cells, re-stimulating the auto-reactive memory-T-cells invading through the blood–brain barrier (BBB) into the CNS. This is considered a key event in maintaining the chronic inflammation in the CNS. [4,5] In active WM lesions, there is a profound infiltration of activated microglia and blood-borne macrophages throughout the lesion, whereas in slowly expanding (smoldering) or chronic active lesions, the microglia and macrophages are concentrated as a dense rim

around the lesions. [6] Microglia have also been found diffusely in WM and GM with concomitant axonal degeneration and meningeal inflammation. [7-9] Thus, chronic activation of microglia has been linked to neurodegeneration in the progressive phase of the disease. [7-9] In progressive MS disease, activation of microglia has been detected in areas of the brain which appear normal in conventional MRI, the so called normal appearing (NA) WM (NAWM) and NAGM. [10] When microglia are activated following an insult, they become mobile and capable of phagocytosis and antigen presentation. The phagocytosis of debris by microglia is thought to be essential for enabling the return of normal tissue homeostasis after an insult. In chronic neuroinflammation, the equilibrium between debris-clearing and inflammation-propagating microglia becomes unbalanced, and the hyper-activated microglia cause uncontrolled neuronal damage resulting in a self-propagating vicious cycle and progressive neurodegeneration. [11] It is thus possible that prolonged inflammation related to activated microglial is one of the driving forces of the chronic progression of MS disease.

Imaging of microglia activation using positron emission tomography:

Positron emission tomography (PET) imaging enables molecular and neuroreceptor imaging in vivo. Particularly relevant for the study of MS is PET imaging of activated microglia. Here, several radioligands binding to the 18 kDa translocator protein (TSPO)—a protein structure expressed on the outer mitochondrial membrane of activated, but not resting microglia—have been developed. [12] Activated microglia express TSPO, earlier called the peripheral benzodiazepine receptor (PBR) on the outer membrane of mitochondria. Several functions have been attributed to TSPO, either directly or indirectly, including immunomodulation, regulation of cholesterol transport, synthesis of steroid hormones, apoptosis, cell proliferation, porphyrin and anion transport, regulation of mitochondrial functions and heme synthesis. [13] Two archetypal ligands binding to TSPO have been identified, namely Ro5-4864, the 4-chloro-derivative of diazepam, and PK11195, the isoquinoline carboxamide, [14] the latter having been used as a marker for in vivo PET imaging of activated microglia. Numerous second-generation TSPO radioligands with higher affinity and specificity than their predecessor [11C](R)-PK11195 have been recently developed.[12,15] ^{18}F-PBR06 is a second-generation TSPO ligand with promising imaging characteristics. [16-19] Recent studies showed increased ^{18}F-PBR06 uptake corresponding specifically to activated microglia in animal models.[20-22] A robust approach for quantitative in vivo assessment of specific ^{18}F-PBR06 binding to TSPO has recently been developed.[23,24] The Singhal et al. study demonstrated that quantitative TSPO PET with a second-generation ^{18}F-PBR06 can be used to characterize innate immune responses in MS in vivo and provides further evidence supporting an association between the WM TSPO PET signal in lesions and disease severity. [23] This approach is practical for extension to studies of the role of the innate immune response in MS for differentiation of anti-inflammatory effects of new medicines and their longer-term impact on clinical outcome.

Rationale and potential benefits

There has been an increasing recognition of the role of B-cells in the pathogenesis of MS since the initial description of intrathecal immunoglobulin synthesis in 1950. In MS, B-cells are present in parenchymal

lesions, tertiary follicle like structures in the meninges and cerebrospinal fluid where they are known to undergo clonal expansion. B-cell directed therapies using anti-CD20 antibodies have been shown to be highly efficacious in preventing relapses in RRMS and slowing progression in primary progressive MS although their mechanisms of action are not clear.[25,26] In animal models, targeting B-cells using antiCD20 antibodies led to reduction in microglial activation and lesion formation.[27] In fact, there was greater effect of anti-CD20 antibodies on microglial activation and lesion formation in the antibody independent model implying that the mechanism of action of these therapies extends beyond antibody production and may involve their actions on microglia.[27] However, there is no human data to date exploring this hypothesis. Ofatumumab is a fully human anti-CD20 antibody that has recently been studied for treatment of relapsing MS in multiple phase II and phase III randomized controlled trials (ASCLEPIOS I and II).

Prior studies using Ofatumumab have demonstrated the time course of B-cell depletion in the periphery. There was 50% reduction by week 2, and >80% reduction by week 4 after administration of Ofatumumab at various doses.[25] Ofatumumab demonstrated high efficacy for relapse rate reduction, new lesion formation and serum neurofilament light chain level reduction in multiple sclerosis patients as compared to an active comparator arm of teriflunomide (Aubagio)[25,28,29]. Additional secondary endpoints were also met, where ofatumumab showed a relative risk reduction in both 3-month confirmed disability (CDP) and in 6-month CDP versus teriflunomide in a pre-specified pooled analysis. Ofatumumab was found to be safe in MS patients.[25,28,29]

However, the effects of Ofatumumab on microglia in the brain in MS patients are not known at this time. It is important to study the effect of anti-CD20 therapies on the innate immune system to understand their role in limiting disease progression in MS. We aim to assess the relationship between peripheral B-cell depletion and changes in microglial activation, after administration of Ofatumumab.

We aim to assess the effect of Ofatumumab on microglial activation using [F-18]PBR06 PET in MS patients in relation to changes in serum markers, MRI abnormalities and clinical impairment longitudinally over 9 months.

Specific Aims:

Specific Aim 1: To determine the effect of Ofatumumab on microglial activation in MS over 9 months.

Specific Aim 2: To determine the time course of effect of Ofatumumab on microglial activation and its relationship with peripheral B-cell depletion, serum neurofilament light (sNfL) chain and glial-fibrillary acid protein (GFAP) levels and other serum biomarkers

Specific Aim 3: To determine the relationship of PET changes following Ofatumumab initiation with 3T MRI changes and clinical parameters.

Design:

This is an open-label, observational, prospective, 9-month follow-up study to assess the efficacy of ofatumumab on microglia pathology in patients with MS, as measured by changes in microglial activation in the lesional and non-lesional, normal appearing white matter, cortical and subcortical grey matter, and peri-plaque area of chronic lesions in the brain.

Initial Visit:

During the first visit, subjects will be administered the screening questionnaire (if that has not already been done over telephone). Subjects will review and eventually sign the consent form. They will be administered a physical examination, clinical assessment and standardized questionnaires for cognitive testing and/or other co-morbidities. In addition, blood samples will be drawn for genotype testing, infection screening, complete blood count and liver function test.

Demographics, physical examinations and neurologic assessments will be conducted at Brigham MS Center, Brigham and Women's Hospital, 60 Fenwood Road, Boston, MA 02115.

Genotype Testing:

Blood sample drawn on the initial screening visit will be used to obtain genomic DNA for genotyping for polymorphism within the TSPO gene on chromosome 22q13.2, using a Taqman assay. High affinity and medium affinity binders will be included in the study, whereas low affinity binders will be excluded from the study.

PET Scanning:

For this study, all subjects will undergo five separate visits for [F-18]PBR06 PET scans, one visit before starting Ofatumumab treatment (day 0 baseline) and four more visits at approximately 5, 28, 90 and 273 days after initiating treatment. During the PET scan visits, all women subjects of child bearing age will undergo a stat quantitative serum hCG pregnancy test and only women with a negative test will undergo the radiopharmaceutical injection. The radiotracers will be produced using standardized procedures. At the time of imaging, the subjects will be positioned in the gantry of a high-resolution PET/CT camera. Head alignment will be made, relative to the canthomeatal line, using projected laser lines whose positions are known with respect to the slice positions of the scanner. A head support apparatus will be used to minimize head motion. Dynamic data over 120 minutes for PET quantification will be acquired, according to previously described methods for the tracer.

MRI Scanning:

All subjects will undergo five 3T brain MRIs with and without contrast, one before starting Ofatumumab treatment (day 0 baseline) and four more at 5, 28, 90 and 273 days after initiating treatment. All women will be queried about their pregnancy status, use of contraception and last menstrual period. If a woman is of child bearing potential, she will undergo a urine pregnancy test. Subjects will undergo intravenous gadolinium contrast administration, during all visits. Hence, all women of child bearing potential will undergo urine pregnancy testing for this study.

Clinical Follow-up Visits:

Subjects will come in for five clinical visits, one visit before starting Ofatumumab treatment (day 0 baseline) and four more visits at 5, 28, 90 and 273 days after initiating treatment. However, if the patient meets all inclusion criteria at the screening visit, the screening and baseline visits may occur on the same day. During these visits, subjects will undergo a physical examination, clinical assessment and cognitive assessment. A blood sample will be drawn to determine levels of sNfL, GFAP and other serum biomarkers. Visits will be conducted under the supervision of PI and other board-certified neurologists. Any adverse event reported by the patient or observed by the investigator will be recorded and reviewed at each clinical visit.

Clinical Data:

The following non-imaging, clinical data will be obtained:

Expanded Disability Status Scale (EDSS), Timed 25-feet walk (T25W), 9-Hole Peg Test (9HPT), Four component MS Functional Composite (MSFC-4), Symbol-Digit Modality test (SDMT), cognitive and symptom questionnaires, vision testing, Levels of serum biomarkers.

Clinical Safety Monitoring:

Safety will be assessed at 0, 3, 6, and 9 months of the study including:

- Pregnancy Testing: All women of childbearing potential will undergo a serum pregnancy test at the screening visit and at each of the following scheduled visits to the hospital. The investigator will also review the contraception status of subjects at each visit.
- Electrocardiogram (ECG): An ECG will be performed either at the screening visit or on day 0 clinical visit and at the end of the study.
- Routine labs: Blood samples will be collected at the screening visit and at each visit time point. Blood samples will be assessed for red blood cell count, hemoglobin, hematocrit, platelets, total white blood cell (WBC) count and WBC differential counts (neutrophils, lymphocytes, basophils, eosinophils, monocytes) as well as for electrolytes (Na, K, Cl, bicarbonate, Ca, Mg, P), random glucose, total protein, blood urea nitrogen, albumin, alkaline phosphatase, ALT, AST, GGT, total bilirubin, conjugated bilirubin, creatinine, amylase, C-Reactive protein.
- Samples for total IgM and IgG levels at baseline, 3, 6 and 9 months
- Patient diary review
- Monthly telephone interview

Drug Administration/Dosing:

At the end of the baseline clinic visit, subjects will be instructed by one of the investigators on proper drug administration technique. The first injection of ofatumumab will be performed under the guidance of an appropriately trained healthcare professional in the clinical space at the Brigham MS Center.

We plan to enroll 10 subjects with relapsing MS. All enrolled subjects will receive Ofatumumab 20 mg every 4 weeks, subcutaneously for 9 months during the study. Loading doses will be administered initially at 1, 7 and 14 days. During the study period, all enrolled subjects will undergo five PET scans using [F-18] PBR06 at 0, 5, 28, 90 and 273 days after starting treatment with Ofatumumab.

Ofatumumab (OMB157) is a fully human anti-CD20 monoclonal antibody (mAb) self-administered by a once-monthly subcutaneous injection that is in development for MS. Ofatumumab drug product (also referred as OMB157) is formulated as 20 mg/0.4 mL (50 mg/mL) solution for injection, provided in autoinjectors, for subcutaneous administration. The autoinjectors contain a small overfill to allow for a complete withdrawal of the labeled amount (20 mg) of ofatumumab.

Inclusion Criteria:

- Patients diagnosed with active, relapsing MS course (defined by Lublin 2014 criteria)30. Active disease is defined by at least 1 relapse during the previous 1 year or 2 relapses during the previous 2 years or a positive gadolinium-enhancing MRI scan or MRI scan with new or unequivocally enlarging T2 lesions in previous year.
- Age 18 to 60 years
- EDSS 0 to 5.5
- Subjects either untreated or treated with disease modifying therapies other than those listed in exclusion criteria
- Agree to start treatment with ofatumumab and comply with study procedures for the duration of the study
- No other systemic disease or neurological disorders requiring chronic or acute steroid or other immunosuppressive treatment
- No known hypersensitivity reactions to contrast agents
- None of the exclusion criteria

Exclusion Criteria:

- Subjects suspected of not being able or willing to cooperate or comply with study protocol requirements in the opinion of the investigator.
- Subjects with primary progressive MS (Polman et al 2011)32 or SPMS without disease activity (Lublin et al 2014)30.
- Disease duration of more than 10 years in patients with an EDSS score of 2 or less
- Subjects meeting criteria for neuromyelitis optica (Wingerchuk et al 2015)33.
- Pregnant or nursing (lactating) women, where pregnancy is defined as the state of a female after conception and until the termination of gestation, confirmed by a positive hCG laboratory test.
- Women of child-bearing potential, defined as all women physiologically capable of becoming pregnant, unless they are using highly effective methods of contraception during dosing and for at least 6 months after stopping study medication. Highly effective contraception methods include:
- Total abstinence (when this is in line with the preferred and usual lifestyle of the subject, if accepted by the local regulation). NOTE: Periodic abstinence (e.g., calendar, ovulation, symptothermal, post-ovulation methods) and withdrawal ARE NOT acceptable methods of contraception
- Female sterilization (have had surgical bilateral oophorectomy with or without hysterectomy), total hysterectomy or tubal ligation at least six weeks before taking investigational drug. In case of oophorectomy alone, only when the reproductive status of the woman has been confirmed by follow up hormone level assessment
- For female subjects on the study, the vasectomized male partner should be the sole partner
- Use of oral, (estrogen and progesterone), injected or implanted hormonal methods of contraception or placement of an intrauterine device (IUD) or intrauterine system (IUS) or other forms of hormonal contraception that have comparable efficacy (failure rate <1%), for

example hormone vaginal ring or transdermal hormone contraception. In case of use of oral contraception, women should have been stable on the same pill for a minimum of 3 months before taking investigational drug.

- In case local regulations deviate from the contraception methods listed above, local regulations apply and will be described in the ICF.
- Women are considered post-menopausal and not of child bearing potential if they have had 12 months of natural (spontaneous) amenorrhea with an appropriate clinical profile (e.g. age appropriate, history of vasomotor symptoms) or have had surgical bilateral oophorectomy (with or without hysterectomy), total hysterectomy or tubal ligation at least six weeks. In the case of oophorectomy alone, only when the reproductive status of the woman has been confirmed by follow up hormone level assessment is she considered not of child-bearing potential.
- Subjects with active chronic disease (or stable but treated with immune therapy) of the immune system other than MS (e.g. rheumatoid arthritis, scleroderma, Sjögren's syndrome, Crohn's disease, ulcerative colitis, etc.) or with an immunodeficiency syndrome.
- Subjects with a history of the following:
  - History of any malignancy
  - History of alcohol or drug abuse
  - Primary or secondary immunodeficiency
  - Prior hematopoietic stem cell transplantation
  - History of transplantation or anti-rejection therapy
- Subjects with the following laboratory abnormalities:
  - Abnormal CD19+ B-cell levels at screening (defined as CD19 count <110 cells per microliter)
  - Leukopenia (defined as white blood cell (WBC) count <4000 WBCs per microliter)
  - Lymphopenia (defined as lymphocyte count <1000 lymphocytes per microliter)
  - Hypogammaglobulinemia defined as a level of <500mg/dL will be excluded. All subjects with low serum immunoglobulins should be evaluated by a hematologic expert prior to exclusion in the study.
  - Any abnormality of liver function tests, including ALT/SGPT, AST/SGOT, Alkaline phosphatase, total or direct bilirubin or GGT
- Subjects with active systemic bacterial, viral or fungal infections, or known to have acquired immunodeficiency syndrome (AIDS).
- Subjects with neurological symptoms consistent with PML or confirmed PML.
- Subjects at risk of developing or having reactivation of syphilis or tuberculosis (eg subjects with known exposure to, or history of syphilis, or active or latent tuberculosis, even if previously treated).
- Subjects with low affinity binders (LAB) for TSPO radioligand
- Subjects with abnormal serum creatinine levels (defined as >1.3mg/dL) or Subjects with estimated glomerular filtration rate (eGFR) <30ml/minute
- Patients with history of significant renal disease (dialysis, kidney transplant, single kidney, renal cancer, renal surgery)
- Patient presenting with cardiac disorders defined by at least one of the following conditions:
- Patient with recent cardiac history (within 6 months) of:
  - Acute coronary syndrome
  - Acute heart failure (class III or IV of the NYHA classification)

- History of significant ventricular arrhythmia (persistent ventricular tachycardia, ventricular fibrillation, resuscitated sudden death)
- Patient with history of cardiac failure class III or IV of the NYHA classification
- Patient with history of severe conduction disorders which are not prevented by permanent pacing (atrio-ventricular block 2 and 3, sinoatrial block)
- Syncope without known etiology within 3 months
- Uncontrolled severe hypertension, according to the judgment of the investigator, or symptomatic hypertension
- Subjects with any contraindications to PET/CT or MRI procedures (e.g. claustrophobia, MRI-incompatible implants or pacemakers, renal failure)
- Subjects with any significant or uncontrolled medical comorbidity
- Subjects with active hepatitis B
- Subjects at risk of hepatitis B reactivation (such as subjects with positive HBsAG or anti-Hepatitis B core antibodies) should be evaluated by a liver disease expert before inclusion, or should be excluded
- Subjects treated with other disease modifying treatments within their respective pre-specified washout periods will be excluded

Biostatistical Analysis

Study endpoints/variables

The primary endpoint of the study will be the change in PET uptake in the lesional and normal appearing white matter, cortical and subcortical grey matter, and peri-plaque area of chronic lesions in the brain at early and late time-points (5, 28, 90 and 273 days) as compared to baseline.

The secondary endpoints of the study will be the following:

1) Association of change in PET uptake in the lesional and normal appearing white matter, cortical and subcortical grey matter, and peri-plaque area of chronic lesions in the brain with changes in the peripheral CD19 counts, serum glial fibrillary acid protein (GFAP) and serum neurofilament light chain measurements at 5, 28, 90 and 273 days as compared to baseline.

2) Association of changes in PET uptake in the lesional and normal appearing white matter, cortical and subcortical grey matter, and peri-plaque area of chronic lesions in the brain with MRI and clinical changes at 5, 28, 90 and 273 days as compared to baseline.

Sample size and statistical considerations:

We will recruit 10 subjects, and each subject will undergo PET evaluations at day 0 (baseline), day 5, day 28, day 90 and day 273. The change with time in PET uptake in the lesional and normal appearing white matter, cortical and subcortical grey matter, and peri-plaque area of chronic lesions will be estimated using an analysis of response profiles model including a categorical effect of time and an unstructured covariance matrix for the residuals. This approach allows any change with time and places no structure on the covariance matrix. The primary analysis will be the global comparison across the five time points. If a significant difference among the five time points is observed, we will estimate the

change between day 0 and each of the other measurements, and we will correct for four multiple comparisons using a Bonferroni correction. Since the analysis of response profiles model estimates the parameters using maximum likelihood with the *mixed* command in Stata, this approach will handle missing data that is missing at random. Therefore, subjects who are missing one or more measurement will be included in our analysis. A p-value <0.05 will be considered statistically significant.

In addition to the primary analysis of the change with time, we will also compare the change in PET uptake with the change in other measured variables (MRI, clinical, and immunologic measures). All of the remaining variables will be measured at the same time points as PET uptake (days 0, 5, 28, 90 and 273). First, we will calculate the change from day 0 to each of the other time points for each of the measures, and we will estimate the correlation between the changes using Pearson's correlation coefficient. This analysis will fit several models, but the interpretation of these correlations will be easy to interpret. Second, we will fit a linear mixed model with PET uptake as the outcome and two predictors for each of the other measured variables. In particular, we will include (1) the day 0 measure and (2) the change from day 0 so that we can estimate both the cross-sectional and longitudinal association between the other measured variables and PET. Our focus will be the longitudinal association estimated using the second term. If a subject receives corticosteroids during the study period, we'll account for that in a multivariate analysis by adding it as a covariate.

Paired t-test and repeated measures ANOVA analyses will be performed to assess differences in PET measures over time. Correlation analyses will be performed between PET and MRI measures and clinical variables. We will also look for correlations between changes in PET uptake and peripheral CD19 counts at various time points of the study.

Statistical analyses will be performed using Stata version 16 (StataCorp, College Station, Texas, USA).

Sample size calculation:

No prior data regarding effect of Ofatumumab on microglial changes is available at this time. The sample size of 10 is based on the typical number of patients in published PET-MRI studies with the same tracer. With a sample size of 10 subjects, we will be able to estimate the mean change between any pair of time points with a standard error of 0.32 times the standard deviation of the change. Assuming a PET effect size of 1.1 noticeable in intracortical and leukocortical lesions[11], identified on combination of PET and 3T MRI, a sample size of 10 provides 85% power and alpha-error of less than 5% to detect a treatment response. This means that we will have a reasonable precision for the design of future studies.

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