JRMO Research Protocol for Interventional Studies

Full Title	Oral Cladribine B-cell study
Short Title	**Clad-B study**
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REC Reference	*<REC Reference number>*
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1. Contents

2. Glossary

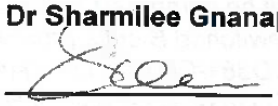
9-HPT	*9-Hole Peg Test*
AE	*Adverse Event*
AR	*Adverse Reaction*
BBB	*Blood Brain Barrier*
CD	*Cluster Differentiation*
CI	*Chief Investigator*
Clad	*Cladribine*
CRF	*Case Report Form*
CSF	*Cerebrospinal Fluid*
CXCL-13	*Chemokine-13*
EDSS	*Expanded Disability Status Scale*
FACS	*Fluorescence-activated cell sorting*
FBC	*Full blood count*
FLC	*Free Light Chains*
GAP-43	*Growth Associated Protein-43*
GFAP	*Glial Fibrillary Acidic Protein*
IgG	*Immunoglobulin G*
JRMO	*Joint Research Management Office*
LFT	*Liver Function Test*
MSFC	*Multiple Sclerosis Functional Composite*
MSIS-29	*Multiple Sclerosis Impact Scale-29*
NCAM	*Neural Cell Adhesion Molecule*
NEDA	*No Evidence of Disease Activity*
NF	*Neurofilament*
OCB	*Oligoclonal Bands*
PROMS	*Patient reported outcome measures*
PBMC	*Peripheral Blood Mononuclear Cells*
REC	*Research Ethics Committee*
RRMS	*Relapsing Remitting Multiple Sclerosis*
SAE	*Serious Adverse Event*
SAP	*Statistical analysis plan*
SAR	*Serious Adverse Reaction*
SDMT	*Single Digit Modalities Test*
SOP	*Standard Operating Procedure*
SUSAR	*Suspected Unexpected Serious Adverse Reaction*
T25FW	*Timed 25 Foot Walk*
U&E	*Urea & Electrolytes*
WCC	*White Cell Count*

3. Signature page

Chief Investigator Agreement

The study as detailed within this research protocol will be conducted in accordance with the principles of Good Clinical Practice, the UK Policy Framework for Health and Social Care Research, and the Declaration of Helsinki and any other applicable regulations. I agree to take responsibility for the statistical analysis and oversight of this study.

Chief Investigator Name: **Dr Sharmilee Gnanapavan**

Signature: 

Date: 21/3/19

4. Summary and synopsis

Short title	*Clad-B Study*
Methodology	*Longitudinal study*
Research sites	*Barts Health NHS Trust* *Queen Mary, University of London*
Objectives / aims	*Primary: To quantify the temporal changes of memory B cells (CD19+/CD27+/IgD-/+), plasmablasts (CD19-/CD138+/CD38+) and T cells (CD4/CD45RA-/+, CCR7-/+, CD8+/CD45RA-/+/CCR7-/+), Tregs (CD4/CD8)/CD25+/CD127-/Fox3 P+) in the peripheral venous blood of pwMS with RRMS over 96w of treatment with oral cladribine.* *These will be compared to the populations of non-memory or class-switched B cells (immature/transitional B cells CD10+/CD38+/CD19+, immature regulatory B cells CD10+/CD38+/CD19+/CD24+/IL-10+, mature B cells CD10-/CD38+/CD19+).* *Secondary:* *1. To study the effects of oral cladribine on:* *a. CSF OCBs and free immunoglobulin kappa and lambda light chain levels (FLC).* *b. CSF markers of inflammation, in particular CXCL-13 and urine markers of inflammation (neopterin).* *c. CSF markers of neuroaxonal damage, in particular free neurofilament light chains.* *d. On the peripheral repertoire B-cells (immunoglobulin) and T-cells (T cell receptor) and plasma cells (soluble receptors).* *2. To compare CSF OCB positivity and CSF light chain levels with a contemporary control group of alemtuzumab treated pwMS (historical data).* *Tertiary:* *1. To compare B and T cell repertoire with a contemporary control group of alemtuzumab treated pwMS (historical data).* *2. To evaluate the effect of changes in the immune cell profile on clinical measures of disability, MRI activity and PROMS.*
Number of participants	*13*
Inclusion and exclusion criteria	*Inclusion:* ● *Patients with RRMS who are being treated with oral cladribine at Barts Health NHS Trust*

	• *Patients must be willing and able to undergo lumbar punctures* • *Patients who are OCB positive in their CSF (from previous diagnostic lumbar puncture)* *Exclusion:* • *Ineligible for oral cladribine under NHS England prescribing guidelines and those participating in MAGNIFY-MS study (cladribine tablets in active MS)* • *Unsuitable to have a lumbar puncture (e.g. spinal deformity, tethered cord syndrome, or the use of aspirin or anticoagulants), and those unable to comply with study requirements, including frequency of visits and lumbar punctures* • *Presence of comorbidities in which administration of cladribine is contraindicated* • *Abnormal baseline investigations (WCC<3 x 10*9/l, neutrophil count < 1.5 x 10*9/l, platelet count < 100 x 10*9/l, haemoglobin < 110 g/l, LFT >/= 3x upper limit of normal site reference ranges, potassium < 2.8 mmol/l or > 5.5 mmol/l, sodium <125 mmol/l, creatinine > 130 umol/l*
Statistical methodology and analysis (if applicable)	*Changes in total immune cell counts will be analysed using descriptive statistics, including reduction relative to baseline and absolute reduction in numbers. Time to nadir and recovery will be done using best-fit modelling.* *Total blood IgG and free kappa and lambda light chain levels will be normalised to albumin levels. McNemar's test will be used to compare differences in OCB status between baseline, year one or year two. The association between different biomarkers, clinical and imaging parameters will be analysed using multiple regression.*
Study duration	*96 weeks.*

5. Introduction

There is emerging data that suggests that B cells, in particularly memory B cells, and intrathecal plasma cell responses in multiple sclerosis (MS) are pathogenic and the intrathecal component is one of the proposed mechanisms that drives delayed non-relapsing progressive MS. As cladribine penetrates the central nervous system, and might have activity against both B-cells and plasma cells, we hypothesise that cladribine may be superior to other disease-modifying therapies by targeting pathogenic intrathecal B-cells and plasma cells. We are particularly interested in see if cladribine targets the intrathecal long-lived plasma cells and the oligoclonal IgG bands (OCBs) they produce, since OCBs have, to date, proven resistant to current treatments including high efficacy treatments such as alemtuzumab. In addition, we will study the effect of cladribine on intrathecal inflammatory (CXCL13) and neurodegenerative (neurofilament light chain) markers. As recent evidence supports the hypothesis that cladribine is working via memory B cells, we will therefore assess the impact of oral cladribine on the kinetics of different peripheral B and T cell depletion and repopulation rates. Finally we will assess the impact of oral cladribine on both the B-cell (immunoglobulin) and T-cell (T cell receptor) repertoire.

5.1 Background

Multiple sclerosis (MS) is invariably associated with an intrathecal oligoclonal IgG response or oligoclonal bands (OCBs) (Giovannoni 2014). This response is not specific to MS and also occurs in central nervous system (CNS) infections and other CNS-specific autoimmune diseases, in particular the paraneoplastic syndromes (McLean, Luxton, and Thompson 1990). Even when the neuroinfectious diseases are successfully treated OCBs persist (van Eijk et al. 1987). In neurosyphilis, for example, OCBs are found a decade, or longer, after successful treatment with antibiotics (“Proteins of the Cerebrospinal Fluid - ScienceDirect” 2016). The persistence of the OCBs has been put down to longevity of long-lived plasma cells (Meinl, Krumbholz, and Hohlfeld 2006). In the case of infections these OCBs target the inciting pathogen and most bands can be removed using antigen-specific immunoadsorption (Luxton et al. 1995). In a putative autoimmune diseases such as MS these OCBs may be targeting self-antigens and may therefore be responsible for driving progressive MS pathology, which theoretically could become independent of autoimmune T-cell help (Meinl, Krumbholz, and Hohlfeld 2006; Giovannoni 2014). Pathological studies have shown that the presence of B-cell-like follicles in the meninges of people with MS (pwMS); these structures are associated with earlier age of onset of progressive disease and earlier age of death (Magliozzi et al. 2007). Recent data has linked the subpial cortical MS lesion with antibody deposition and complement activation (Wolfgang Bruck, 2015; personal communication), similar to that what has been described in the so called type 2 acute MS lesion (Lucchinetti et al. 1996). CSF from pwMS is gliotoxic and neurotoxic in vitro (Øren, White, and Aasly 2001) and in vivo; this toxicity may be antibody mediated. Based on these observations OCBs are likely to be pathogenic in MS. To the best of our knowledge none of the currently licensed disease-modifying therapies have been convincingly shown to eliminate the intrathecal OCBs in pwMS, with the possible exception of natalizumab (von Glehn et al. 2012). There have been a few small case series showing the loss of OCBs in a proportion of patients treated with natalizumab (Mancuso et al. 2014; von Glehn et al. 2012; Krasulová et al. 2007). However, this

observation was not confirmed in a larger, more definitive, German study (Harrer et al. 2013). It is important to note that in pwMS treated with alemtuzumab intrathecal oligoclonal bands persist (Hill-Cawthorne et al. 2012). In comparison, in the Scripps chronic progressive cladribine trial, patients treated with cladribine had a significant decline in the number of oligoclonal bands (Sipe et al. 1994). Although the OCBs did not disappear this study used agarose gel electrophoresis, a technique that is not specific for detecting immunoglobulins and is now obsolete (Freedman et al. 2005).

We hypothesise that it will be important to target intrathecal plasma cells therapeutically to delay the onset, or slow down, the clinically-apparent progressive phase of MS. Most licensed DMTs are effective, to a greater or lesser extent, at suppressing relapses and focal MRI activity (new T2 or Gd-enhancing lesions), however, none of the treatments have yet been shown to prevent the onset of non-relapsing progressive disease. Why? If the non-relapsing progressive phase of the disease is driven by intrathecal OCBs, the resistance of long-lived plasma cells to current therapeutic intervention may explain why this phase of the disease is so resistant to treatment. To successfully treat, or prevent, progressive MS intrathecal plasma cells may need to be eliminated to prevent antibody mediated mechanisms that underlie the progressive phase of MS. We are thus actively exploring treatments that target intrathecal plasma cells; for example, we are proposing that cladribine (2-chlorodeoxyadenosine) a nucleoside analogue penetrates the blood-brain barrier (BBB) and has been shown to have biological activity against myeloma cells, or malignant plasma cells, albeit in combination with a STAT3 inhibitor (Ma et al. 2011).

This suggests that cladribine may target both peripheral and central anti-inflammatory mechanisms including pathogenic intrathecal plasma cells. We hypothesise therefore that cladribine will be effective as a treatment for progressive MS; cladribine by targeting intrathecal plasma cells in MS will reduce intrathecal IgG and free light chain production and may result in the disappearance of intrathecal OCBs. We are therefore proposing an intensive B-cell biomarker study (CLAD-B study) to explore the effect of cladribine on peripheral and intrathecal B cells and plasma cells. As cladribine has a greater impact on peripheral B cells, in particularly memory B cells (B. Ceronie, M. Zhifeng, F. Ammoscato, H. Lock, H.J. Longhurst, G. Giovannoni, D. Baker, K. Schmierer 2017), and to look at the differential effect of cladribine on both the T & B cell repertoire.

We have recently hypothesised that all effective MS DMTs, particularly high-efficacy DMTs, work via targeting the peripheral memory B-cell population and suggested that cladribine may be preferentially working as semi-selective B-cell agent (Baker et al. 2017). We now have cross-sectional pilot data from patients treated with parenteral cladribine showing prolonged peripheral memory B-cell depletion of a similar magnitude to alemtuzumab-treated patients (Baker et al. 2017; B. Ceronie, M. Zhifeng, F. Ammoscato, H. Lock, H.J. Longhurst, G. Giovannoni, D. Baker, K. Schmierer 2017). As part of this study we propose defining in detail the changes in peripheral lymphocyte subsets in both the depletion and repopulation phases after oral cladribine. In addition, we will study the effects of oral cladribine on the peripheral blood B and T cell repertoire by studying the immunoglobulin and T-cell receptor diversity.

5.2 Preclinical data

Intrathecal plasma cell and B cell biology

In MS both quantitative and qualitative assays are used to assess pathological intrathecal IgG production, which is an indicator of intrathecal B cell and plasma cells activity. From a diagnostic perspective qualitative assays are more sensitive being abnormal in 95-98% of patients with clinically definite MS. The Ig-Index, and other similar indices, are less sensitive only being abnormal in ~70% of people clinically-definite MS. However, these indices do provide a proxy of the intrathecal B and plasma cell activity. The downside of using IgG as a marker of activity is its long circulating peripheral half-life of IgG that results in the equilibration of levels between the blood and intrathecal compartments. The equilibration ratio is very sensitive to any disruptions in blood-brain-barrier (BBB) hence the need to adjust CSF:plasma IgG ratio with the albumin quotient, an index of BBB integrity. In comparison free immunoglobulin light chains (FLCs), both kappa and lambda, have a very short circulating half-life due to their high fractional excretion rate in the kidney and catabolism. As a result levels of FLCs between the blood and CSF don't equilibrate. Therefore, CSF FLC levels are a very good proxy of intrathecal B-cell and plasma cell activity. Therefore, any drug that reduces activity of B-cells and/or plasma cells will lower FLC levels. Unfortunately, FLCs don't differentiate between B-cells and plasma cells. We are not aware of any specific soluble CSF biomarker, apart from FACs analysis of free cells in the CSF that can reliably differentiate between B-cells and plasma cells.

The molecular mechanisms underlying terminal differentiation of B cells into plasma cells, and long-lived plasma cells, are partially understood and involve both transcriptional and epigenomic changes. As B cells differentiate into plasmablasts there is a committal step in which an S phase-synchronized differentiation switch is associated with an extensive DNA demethylation and local acquisition of 5-hydroxymethylcytosine at enhancers and genes related to plasma cell identity (Caron et al. 2015). Downregulation of both TGFb1/SMAD3 signaling and p53 pathway support this final step, allowing the emergence of a CD23-negative subpopulation in transition from B cells to plasma cells. Hydroxymethylation of PRDM1, a gene essential for plasma cell fate, is coupled to progression in S phase. DNA methylation patterns can potentially be used to study the B cell maturation process in the context of MS (Kulis et al. 2015) and we propose exploratory epigenetic studies in the future (samples from this study will be stored for this).

B-cells

Preliminary evidence from therapeutic studies suggests that the memory B cell pool might play a crucial role in the disease pathogenesis (Baker, D., Marta, M., Pryce, G., Giovannoni, G., Schmierer, K. 2016). Limited evidence has shown that the memory B cell pool is suppressed by several of the disease modifying therapy (DMT) agents used in RRMS (Kasper et al. 2014)(Thompson et al. 2010)(Rieckmann 2009; Palanichamy et al. 2014). This appears to correlate with disease suppression and the desired clinical outcome of no evidence of disease activity (NEDA)(Coles et al. 2012; Leist et al. 2014; Bar-Or et al. 2008). This suggests that memory B cells may have a role in the mechanisms underlying demyelination in RRMS. It also suggests that they might have utility as a marker for disease suppression. However, the suppression of

memory B cells by DMT, and their contribution to pathogenesis in MS, remains unclear.

The maxim that CD4 Th17 cells play an important role in RRMS pathogenesis is largely based on animal models (Rostami and Ciric 2013; Volpe, Battistini, and Borsellino 2015). However, this failed to be demonstrated in human subjects, where their suppression in clinical trials has only produced marginal benefits (Deiß et al. 2013). It is clear then that other mechanisms must play a crucial role in demyelination and relapse.

Alemtuzumab is an anti-CD52 monoclonal antibody that is one of the most clinically efficacious DMT agents, capable of inducing long-term disease suppression (Coles et al. 2012). Studies of the B cell pool have shown that, while immature B cells rapidly repopulate after therapy, $CD19^+/CD27^+$ memory B cells are significantly depleted for up to 30 months following treatment(Coles et al. 2012; Kasper et al. 2014; Thompson et al. 2010). While alemtuzumab also suppresses $CD4^+$ and $CD8^+$ T cells, changes in the T cell pool do not correlate with disease activity (Bar-Or et al. 2008). Cladribine is a drug originally licensed for hairy cell and B-cell chronic lymphocytic leukaemia. It has also been shown to be highly effective in suppressing disease activity in RRMS (Leist et al. 2014). One report has suggested that it preferentially depletes the CD19 B cell pool, with only modest effects on CD4 and CD8 cells (Leist et al. 2014; Rieckmann 2009). Rituximab is anti-CD20 monoclonal antibody that suppresses T cells by approximately 20-25% (Palanichamy et al. 2014). However, it also produces a sustained depletion in memory B cells that lasts for up to a year, which parallels disease activity (Palanichamy et al. 2014; Bar-Or et al. 2008).

Taken together, these studies suggest that some of the most clinical effective DMT agents MS seem to target the memory B cell pool ($CD19^+/CD27^+$), which advocates for the role of these cells in disease activity (Baker et al. 2017). However, their degree of depletion, what subsets may be implicated, and the exact mechanisms by which they might contribute to pathogenesis, remains to be explored. While the limited reports do suggest the memory B cell pool may be affected, no studies have compared alemtuzumab and cladribine directly in terms of their effect on the compartment, and whether there may be more specific effects of specific subset depletion within the compartment.

Several studies causing B cell augmentation seem to worsen disease activity in MS. Atacicept is a homodimeric fusion protein that stimulates B cell numbers, activation and antibody production (Palanichamy et al. 2014; Bar-Or et al. 2008; Gatto 2008). It was reported to precipitate CNS inflammation and progression to clinically definite MS in a trial of optic neuritis(Sergott et al. 2015) and a clinical study in MS was discontinued after worsening of disease (Sergott et al. 2015; Kappos et al. 2014). Similarly, TNF inhibitors have been shown to augment memory B cell populations in trials of rheumatoid arthritis, sometimes leading to development of CNS demyelinating disease (Roll et al. 2012; Souto-Carneiro et al. 2009). TNF inhibitors have further been shown to worsen the disease course of MS (van Oosten et al. 1997). These data provide some indirect evidence that memory B cells may have an important function the manifestation of disease in RRMS (Baker et al. 2017).

It is unclear what the precise role of memory B cells is in driving CNS inflammation, as they are a heterogeneous population with many different functions. In the progression of disease activity there is a shift in the makeup of unswitched and naïve B cells towards $CD19^+$/CD27+ memory cells (van Oosten et al. 1997; Haas et al.

2011). It is known that they express co-stimulatory molecules and major histocompatibility (MHC) proteins and can present myelin antigens (Michel et al. 2015; Harp et al. 2010). Given this, it has been posited that they may act as important antigen presenting cells to drive T cell mediated mechanisms of inflammation (Baker, D., Marta, M., Pryce, G., Giovannoni, G., Schmierer, K. 2016). If particular, a subset of $CD19^+/CD27^+/CD25^+$ memory B cells, which express the Interleukin 2A Receptor, are increased intrathecally in MS (de Andrés et al. 2014), and appear to have a regulatory function on $CD4^+$ T cells (Kessel et al. 2012). If they play a crucial role in the pathogenic mechanisms of MS, and their activity correlates with disease activity, they might serve as a useful biomarker of disease activity in response to DMT (Baker, D., Marta, M., Pryce, G., Giovannoni, G., Schmierer, K. 2016; Komori et al. 2015). Thus, it is of great clinical interest to characterise which of these cells and their myriad subsets are most closely related to disease suppression.

Memory B cells are a heterogeneous group of lymphocytes (Michel et al. 2015). Which specific subsets are most closely related to disease activity requires greater elucidation. Important subsets include the class-switched IgD^+ and non class-switched IgD- subtypes. Circulating plasmablasts, which co-express the $CD19^+/CD27^+/CD38^+$ marker set, may also be of clinical importance, as their levels are increased throughout the course of MS (Michel et al. 2015; Cepok 2005). Only one study has examined a pathogenic subset of $CD27^+/CD38^{low}$ memory B cells in MS, which are increased in untreated disease (Li et al. 2015). However, no previous study has examined the activity of memory B cell subsets in response to therapy. We would therefore like to characterise not only the general $CD19^+/CD27^+$ pool, but also subsets of $IgD^\pm$ and $CD25^\pm$ memory B cells and $CD38^+$ plasmablasts. This may give us greater insight into the effects of DMT and the role of B cells subsets in RRMS.

Limited therapeutic evidence supports the notion that $CD19^+/27^+$ memory B cells may play a vital role in contributing to the disease pathophysiology of MS (Kasper et al. 2014) (Thompson et al. 2010; Rieckmann 2009; Palanichamy et al. 2014). Moreover, CD27 is associated with immunoglobulin index in cerebrospinal fluid in MS (Komori et al. 2015). Their association with disease activity, and suppression by effective DMT, suggests that they might play a vital role in MS pathogenesis. If $CD19^+/CD27^+$ memory B cells are proven to be key protagonists in the disease process, they might be valuable targets for the development of novel therapeutic agents that specifically target the memory B cell pool.

5.3 Rationale

Study objectives: To study the impact of cladribine on peripheral and intrathecal B-cell, plasma cells, T cells and Tregs.

While some treatments have been shown to affect this pool, it is not clear to what degree, or if there are specific pathogenic subsets that might be interesting drug targets, in order to maximise clinical efficacy while minimising adverse effects. This study will therefore attempt to explore the memory B cell pool and related subsets, plasmablasts and T cell family in order to further characterise their response to therapy.

Hypotheses

1. Oral cladribine preferentially targets memory B cells and that the repopulation of B-cells is driven initially be immature and subsequently mature B-cells, but not memory B cells.
2. In people with MS (pwMS) the administration of cladribine will reduce CSF biomarkers of both B cell and plasma cell activity (OCBs and absolute free light chain levels) and markers of CNS inflammation (CXCL-13) and neuroaxonal damage (neurofilament light chain).
3. The administration of oral cladribine to be pwMS will result in changes, or rejuvenation, of immune repertoire at both a B-cell (immunoglobulin) and T-cell (T cell receptor) level.

5.4 Risks / benefits

There are small risks associated with venepuncture and lumbar puncture that will form the main method of sample collection. These include haematoma formation, infection, nerve damage, haemoconcentration, extravasations, syncope and fainting, petechiae, excessive bleeding, oedema, fear and phobia, thrombosis, arterial puncture, pain and allergy (Campbell, Carrington, and Limber 1999). However, these risks are rare if performed competently. There are no direct benefits to the participants taking part in the study. However, there may be a future benefit to current or future MS patients, depending on the results of the study.

6. Study objectives

6.1 Primary objective

To quantify the temporal changes of memory B cells (CD19+/CD27+/IgD-/+), plasmablasts (CD19-/CD138+/CD38+) and T cells (CD4/CD45RA-/+, CCR7-/+, CD8+/CD45RA-/+/CCR7-/+), Tregs (CD4/CD8)/CD25+/CD127-/Fox3 P+) in the peripheral venous blood of pwMS with RRMS over 96w of treatment with oral cladribine. These will be compared to the populations of non-memory or class-switched B cells (immature/transitional B cells CD10+/CD38+/CD19+, immature regulatory B cells CD10+/CD38+/CD19+/CD24+/IL-10+, mature B cells CD10-/CD38+/CD19+).

6.2 Secondary objective

1. To study the effects of oral cladribine on:

    a. CSF OCBs and free immunoglobulin kappa and lambda light chain levels (FLC).
    b. CSF markers of inflammation, in particular CXCL-13 and urine markers of inflammation (neopterin).
    c. CSF markers of neuroaxonal damage, in particular free neurofilament light chains.
    d. On the peripheral repertoire B-cells (immunoglobulin) and T-cells (T cell receptor) and plasma cells (soluble receptors).

2. To compare CSF OCB positivity and CSF light chain levels with a contemporary control group of alemtuzumab treated pwMS (historical data).

6.3 Tertiary objective

1. To compare B and T cell repertoire with a contemporary control group of alemtuzumab treated pwMS (historical data).

2. To evaluate the effect of changes in the immune cell profile on clinical measures of disability, MRI activity and PROMS.

6.4 Primary endpoint

The time course of B, plasmablast and T cell depletion and re-population over 96w to see if any particular population of cells is more susceptible to cladribine, i.e. memory B-cells ($CD19^+/CD27^+$). These changes will be compared to populations of naïve B cells.

6.5 Secondary endpoint

1. a. Relative change in CSF OCB pre- and post-cladribine (baseline *vs* 48w and 96w).
b. Relative change in CSF cytokines and chemokines (baseline *vs* 48 w and 96w period), in particular CXCL-13 pre- and post-cladribine and urine neopterin levels.
c. Relative change in CSF neurofilament light chain pre-and post-cladribine (baseline *vs* 48w and 96w).
d. Relative change in blood immunoglobulin levels and T cell receptor levels (baseline *vs* 48 w and 96w period) pre-and post-cladribine and urine immunoglobulin levels.
2. Relative change in CSF soluble CD138 (plasma cell marker) pre- and post-cladribine (baseline *vs.* 48w and 96w).
3. Occurrence of CSF OCB positivity and relative change in CSF FLC post-cladribine *versus* historical data post-alemtuzumab (baseline *vs*. 48w and 96w).

6.6 Tertiary endpoint

1. Relative change in B and T cell repertoire post-cladribine *versus* historical data post-alemtuzumab.

2. Correlation between baseline, 48w and 96w immune cell profiles and baseline, 48w and 96w EDSS, MSFC, T2 lesion load and gadolinium enhancement, MSIS-29 and their changes.

7. Study population

This longitudinal study over 96 weeks will examine peripheral venous blood samples, CSF and urine of pwMS with RRMS who are treated with oral cladribine tablets under the NHS in Barts Health. Only those able to give consent to participate in the study will be included.

7.1 Inclusion criteria

1. Patients with MS who are being treated with oral cladribine at Barts Health NHS Trust will be approached to participate in this study.
2. Patients must be willing and able to undergo lumbar punctures.
3. Patients who are OCB positive in their CSF (previous diagnostic lumbar puncture).

7.2 Exclusion criteria

1. Ineligible for oral cladrabine under NHS England prescribing guidelines and those participating in MAGNIFY-MS study (cladribine tablets in active MS).
2. Unsuitable to have a lumbar puncture, for example spinal deformity, tethered cord syndrome or the use of aspirin or anticoagulants, and those unable to comply with study requirements, including frequency of visits and lumbar punctures.
3. Presence of comorbidities in which the administration of cladribine is contraindicated.
4. Abnormal baseline investigations (WBC<3 x 10*9/l, lymphocytes <1.0 x 10*9/l, neutrophil count <1.5 x 10*9/l, platelet count <100 x 10*9, haemoglobin <110g/l, LFT>/3x upper limit of normal of site reference ranges, potassium <2.8mmol/l or >5.5mmol/l, sodium <125 mmol/l, creatinine >130 umol/l).

8. Study design

Patients will be required to give consent for 15mL of peripheral venous blood (plasma and serum), urine and CSF to be collected at multiple time points over 96 weeks. These samples will be collected in addition to those collected for routine clinical monitoring. They will be informed that their decision to consent for their blood products to be used in research will have no impact on their current or future treatment, and it should not affect their decision to start, continue or discontinue their MS therapy.

Venous blood samples will be analysed by flow cytometry. B cell populations will be determined using specific markers for B cell populations including CD10, CD19, CD25, CD27, CD38 and IgD. This will allow the analysis of the number of memory B cells ($CD19^+/CD27^+$), memory B cell subsets ($CD19^+/CD27^+/IgD^{-/+}$ and $CD19^+/CD27^+/CD25^{-/+}$) and plasmablasts ($CD19^+/CD27^+/CD38^+$) in the subject's blood samples. As controls, naive B cells CD19+/CD27- and T cells ($CD19^-$

/CD3$^+$/CD4$^+$/CD8$^+$) will also be examined. Whole blood for DNA extraction will be collected and stored for deep sequencing to analyse the B and T cell repertoire using Adaptive Biotechnologies' immunoSEQ technology; these assays can identify millions of T- and B-cell receptors in a single sample. Using this technology will allow us to compare the impact of cladribine on the adaptive immune system over time and to compare it with similar data from alemtuzumab treated subjects from Prof. Heinz Wiendl's laboratory in Munster.

CSF will be collected using an atraumatic needle. CSF will be processed for routine biochemistry and aliquots will be stored for OCBs, FLC, B cells: soluble CD19 and plasma cells: soluble CD138; inflammatory markers: urine neopterin and multiplex cytokine profile; and neurodegenerative markers: NF, GFAP, NCAM, GAP-43 and S100B levels.

Study subjects will also be examined annually, have a routine annual MRI T2 lesion load and gadolinium enhancement, and the following outcome measures assessed EDSS, 9-HPT, T25FW, MSIS-29 and other routine clinical outcome measures at 24 weekly intervals.

Historical control data from 12 post-alemtuzumab treated subjects (Barts Health) for secondary endpoints (CSF OCB & FLC) and tertiary endpoints (B and T cell repertoires) will be used as comparators.

9. Study procedures

Participants will be counselled on the risks of the treatment and any study procedures (bloods, urine sampling and lumbar punctures) when obtaining full informed consent, and will specifically exclude those who may be vulnerable to these risks. Informed consent to participate in the study will be taken by the research fellow, treating neurologist or the study CI (Dr Sharmilee Gnanapavan). It will be explained to all participants that they are free to withdraw their consent at any time without needing to provide an explanation, and their medical care will not be affected in any way if they decide not to participate. The data collected up to the point of withdrawal will be included in the analysis at the end.

All serious adverse events will be reported to the Sponsor and the research ethics committee by the project manager and PI (see Section 17: safety reporting). Clinical databases will be maintained by the project manager and access to personal data will be limited to the study team, and all other data will otherwise be anonymised. Day to day management of the study including MRI evaluation will be handled by the research fellow in conjunction with the neuroradiology department at Barts Health NHS Trust. Clinical scores, EDSS and timed 25 foot walk, and lumbar punctures will be performed by the research fellow or another trained neurologist listed on the delegation log.

Lumbar punctures will be performed using atraumatic needles to minimise the risk of post-puncture headaches at Barts Health NHS Trust day unit in accordance with routine clinical practice. Sample processing and storage (including freezer maintenance) will be performed by the laboratory technician in accordance with Departmental standard operating procedures (SOPs). Laboratory analysis will be performed by the research fellow, as well as the data analysis in conjunction with the study statistician.

Schedule of study interventions:

	Baseline prior to 1	Treatment Phase	Extra																																							
Week	**Baseline - week 0**	**Week 1**	**Day 5**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**	**24**	**28**	**32**	**36**	**40**	**44**	**48**	**52**	**56**	**60**	**64**	**68**	**72**	**76**	**80**	**84**	**88**	**92**	**96**
Estimated Patient num	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Study specific visits			12	12	12		12	12	12		12	12	12	12	12	12	12		12	12	12	12	12			12	12	12	12		12	12	12	12	12	12	12	12	12	12	12
Informed consent	1																																									
History	1																	1						1					1													1	
Examination	1																	1						1					1													1	
EDSS/MSFC/PROMS	1																	1						1					1													1	
Oral Cladribine		1				1																		1	1																		
Labs																																										
FBC	1					1				1								1						1	1				1													1	
U&E	1					1				1								1						1	1				1													1	
LFTS	1					1				1								1						1	1				1													1	
TFTs	1																							1																			
Pregnancy test	1																																									
HIV1&2	1																																									
Hep B&C	1																																									
VZV serology	1																																									
Syphilis serology	1																																									
TB elispot	1																																									
Procedures																																										
MRI	1																							1																			1	
LP	1																							1																			1	
Biobanking																																											
DNA	1																																									
Plasma	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Serum	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cells	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CSF	1																							1																			1	
FAC & Biomarkers	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ImmunoSeq	1																								1																			1	
Urine	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Assumptions are based on a 6 month recruitment period

Routine NHS care
OCB-Specific Study care

10. Assessment and management of risk

Blood tests are a safe procedure, but there can be complications, such as bruising at the site of puncture and excessive bleeding. There is also the risk of needle stick injury to the person performing the test. We will minimise the risk of complications by having a trained staff member perform the blood extraction in accordance with infection control guidelines.

Lumbar puncture will be an aseptic procedure under local anaesthetic performed by a neurologist with experience to minimise the risk of infection and to maintain comfort. Atraumatic needles will be used to reduce the incidence of headache post lumbar puncture secondary to CSF leak. Potential but rare risk of lumbar puncture (approximately 1/10,000) is bleeding and nerve damage. Measures will be taken to minimise bleeding complications by applying pressure to the lumbar puncture site after the needle is removed and by not performing lumbar punctures on those on blood thinners (including aspirin, warfarin or heparin). The use of landmarks to identify site of the lumbar puncture beforehand, as well as partly withdrawing the introducer to check if there is CSF and that needle is in the subarachnoid space will minimise the incidence of nerve injuries. Telephone contact details will be provided as part of aftercare, for either the subject or their partner to access a member of the study team if there are any possible safety concerns.

Samples will be processed according to laboratory SOPs and COSHH procedures to minimise the risk of sample mismanagement and infection risk.

11. Statistical considerations

11.1 Sample size

This is an exploratory study and all analyses are descriptive. The primary endpoint of this study will be to look at the effect of oral cladribine on peripheral immunological markers over time. However, to explore the intrathecal effect of cladribine we will assess its impact on the disappearance of OCBs from the CSF. Based on published data none of the patients with MS will be expected to lose OCBs over 2 years of the study. With a power of 80%, and an alpha of 5%, we will only need 11 subjects to show a significant result if at least 50% of subjects on cladribine become OCB negative compared to a comparator group. Allowing for a 20% dropout rate we would only need to recruit 13 subjects.

11.2 Method of analysis

Depletion and re-population kinetics of B and T cell subsets will be analysed using descriptive statistics. An interim analysis of the year 1 data is planned, and a further analysis of the whole data will be performed at the end of the study. These will be calculated as a reduction relative to baseline counts and the absolute reduction in numbers. Time to nadir and recovery will be done using best-fit modelling. Categorical data based on absolute cut-offs will be presented as proportions, e.g. for absolute lymphocyte counts this will use the WHO boundaries.

Total blood IgG and free kappa and lambda light chain levels will be normalised to albumin levels. McNemar's test will be used to compare differences in OCB status between baseline and year one CSF or year two CSF. Differences in CSF and blood biomarkers, clinical and imaging parameters between baseline and 48w and 96w will be analysed using multiple regression. A detailed SAP will be prepared and approved during the recruitment period well before data becomes available.

12. Ethics

The Principal Investigator will ensure that the study is carried out in accordance with the ethical principles in the Research Governance Framework for Health and Social Care, Second Edition, 2005 and its subsequent amendments as applicable and applicable legal and regulatory requirements. A favourable opinion will be sought from NHS REC.

13. Public Involvement

The therapeutic strategy and concept employed in this trial have been discussed with service users and pwMS during Patient Engagement and focus group events. Results of the study will be shared within these events and also on the MS research blogspot: http://multiple-sclerosis-research.blogspot.com/.

14. Data handling and record keeping

14.1 Data management

Study data will be captured in source documents such as the hospital medical notes, laboratory reports, ECG print-outs and study-specific source document worksheets. Data will be entered into the case report form (CRF) by delegated members of the study team. All data generated by the study will be stored securely with a full audit trail.

14.2 Source data

Data will be recorded from a variety of sources including participant-reported information, physical examination findings, and print-outs from equipment, recording results from electronic displays, laboratory reports and validated questionnaires. All efforts will be made to maximise completeness of data e.g. telephoning subjects. All interactions with participants will be documented (e.g. telephone conversations). Questionnaires will be completed by participants and that every questionnaire should be data and signed by the person completing it. The CI/delegate will keep records of all participants (sufficient information to link records e.g., CRFs, hospital records and samples), all original signed informed consent forms and copies of the CRF pages. All data generated by the study will be stored securely with a full audit trail.

14.3 Confidentiality

Information related to participants should be kept confidential and managed in accordance with the Data Protection Act, NHS Caldecott Principles, The Research Governance Framework for Health and Social Care, and the conditions of Research Ethics Committee Approval.

14.4 Record Retention and Archiving

When the research trial is complete, it is a requirement of the Research Governance Framework and Trust Policy that the records are kept for a further 20 years. For trials involving BH Trust patients, undertaken by Trust staff, or sponsored by BH or QMUL, the approved repository for long-term storage of local records is the Trust Corporate Records Centre. All research documentation will be archived in physical form.

15. Laboratories

15.1 Central and local laboratories

Specimen collection and processing: standard operating procedures will be in place outlining CSF, blood and urine and collection, transport, processing and storage either to the relevant Barts Health NHS Trust laboratory for routine specimens or to the Blizard Institute research laboratories for research samples.

15.2 Sample collection and preparation

Specimen collection, handling and processing will be performed in accordance with written SOPs. Because the adequacy of the specimen will affect the accuracy and usefulness of the biomarker results, all CSF will be processed within an hour of collection, whilst the quality of the blood specimen will be assessed for general interferences, such as haemolysis and lipemia by RLH Pathology Labs. Samples will have a unique identifier with participant details anonymized and stored at -80C until required for analysis.

15.3 Laboratory procedures

Four (serum, whole blood and cells) additional samples of venous blood will be taken during the venepuncture to obtain their routine clinical blood tests. The whole blood will be stored for future ImmunoSEQ analysis. The CSF collection and urine collection would also be an additional tests. Because the adequacy of the specimen will affect the accuracy and usefulness of the biomarker results, all CSF will be processed within an hour of collection, whilst the quality of the blood specimen will be assessed for general interferences, such as haemolysis and lipemia by Barts Health NHS Trust Pathology Labs. Samples will have a unique identifier with participant details anonymized.

PBMC Preparation: Peripheral blood mononuclear cells (PBMCs) will be obtained from venous blood by density gradient centrifugation using Ficoll (Invitrogen). Briefly the blood will be diluted 1:1 in PBS (without Ca2+, Mg2+) and 25ml loaded on Ficoll in a 1:1 ratio. Tubes will then be centrifuged for 30min at 1500rpm at 20^{o}C. The buffy coat will be collected in a 50ml tube and washed twice in PBS. Finally the pellet will be resuspended in 1ml PBS and cells counted with haemocytomer.

Extracellular Staining: Cells will be washed twice with 500ul of FACS buffer (2% FBS, 2mM EDTA in PBS). Cells with then be blocked for 20 minutes in the dark with 100ul of blocking solution (FACS buffer). Cells will then be labelled with antibodies of interest (anti-live, anti-CD3, anti-CD10, anti-CD19, anti-CD25, anti-CD27, anti-CD38, anti-IgD; BD). They will be left for 30 minutes on ice before being washed twice with FACS, then centrifuged for 5 minutes at 1300 and 4^{o}C. The pellet will be resuspended in 200ul of FACS buffer. They will then be analysed.

15.4 Sample storage and transfer

The routine blood tests will be sent to the NHS pathology laboratory at the Royal London Hospital. The additional sample will be transferred by hand to the laboratory at the Blizard Institute. Aside from the PBMCs (see above), samples will be stored at -80C awaiting analysis.

16. Interventions and tools

16.1 Medicinal product

Participants will receive oral cladribine based on licensed indication for cladribine (Mavenclad, Merck Serono) in RRMS (NICE TA493) under the NHS. In the summary of product characteristics the recommended cumulative dose is 3.5 mg/kg body weight over 2 years, taken as 1 treatment course of 1.75 mg/kg per year. Each treatment course consists of 2 treatment weeks, 1 at the beginning of the first month and 1 at the beginning of the second month of the respective treatment year. Each treatment week consists of 4 or 5 days on which a patient takes 10 mg or 20 mg (1 or 2 tablets) as a single daily dose, depending on body weight. Following completion of the 2 treatment courses, no further cladribine treatment is required in years 3 and 4. Cladribine will be stored and dispensed by the Barts Health NHS Trust pharmacy.

17. Safety reporting

Adverse Events (AEs) and Adverse Reactions (ARs) will be recorded. Serious Adverse Events (SAEs) and Serious Adverse Reactions (SARs) will be reported to the CI as medical assessor for the sponsor and coordinating team. All unexpected SARs (SUSARs) will be reported to the JRMO and REC.

17.1 Adverse Events (AEs)

An AE is any untoward medical occurrence in a participant to whom an intervention has been administered, including occurrences which are not necessarily caused by or related to that intervention. An AE can therefore be any unfavourable or unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with study activities.

17.2 Adverse Reaction (ARs)

An AR is any untoward and unintended response in a participant to an intervention. All adverse events judged by either the reporting investigator or the sponsor as having a reasonable causal relationship to the intervention qualify as adverse reactions. The expression ‘reasonable causal relationship’ means in general that there is evidence or an argument to suggest a causal relationship.

17.3 Notification and reporting of Adverse Events and Reactions

If the AE is not defined as serious, the AE will be recorded in the study documents and the participant followed up by the research team. The AE will be documented in the participants' source documents, the Case Report Form (CRF), and, where appropriate, medical records.

17.4 Serious Adverse Events (SAEs) or reactions

A serious adverse event (SAE) is defined as an untoward occurrence that:

- Results in death,
- Is life-threatening,
- Requires hospitalisation or prolongation of existing hospitalisation,
- Results in persistent or significant disability or incapacity,
- Consists of a congenital anomaly or birth defect, or
- Is otherwise considered medically significant by the investigator.

SARs will be reported to the REC where in the opinion of the Chief Investigator the event was serious and:

- Related (it may have resulted from administration of any of the research interventions), and
- Unexpected (the type of event is not listed in the protocol or other Reference Safety Information as an expected occurrence).

17.5 Notification and reporting of Serious Adverse Events

Serious Adverse Events (SAEs) that are considered to be 'related' and 'unexpected' will be reported to the sponsor and the funder of the studied medicinal product within 24 hours of learning of the event, and to the REC within 15 days in line with the required timeframe.

17.6 Urgent Safety Measures

The CI will take urgent safety measures if necessary to ensure the safety and protection of the clinical study participant from immediate hazards to their health and safety. The measures will be taken immediately. The approval of the REC prior to implementing urgent safety measures is not required. However the CI will inform the sponsor and Research Ethics Committee (via telephone) of this event immediately.

The CI will inform the REC in writing within 3 days, in the form of a substantial amendment. The sponsor (Joint Research Management Office (JRMO)) will be sent a copy of the correspondence with regards to this matter.

17.7 Annual Safety Reporting

The CI will send the Annual Progress Report to the REC using the HRA template (the anniversary date is the date on the REC "favourable opinion" letter) and to the sponsor.

17.8 Overview of the Safety Reporting responsibilities

The CI is the medical assessor on behalf on the sponsor and will review all events reported. The CI will ensure that safety monitoring and reporting is conducted in accordance with the sponsor's requirements.

18. Monitoring and auditing

The sponsor or delegate retains the right to audit any study, study site, or central facility. Any part of the study may be audited by the funders, where applicable.

Study will be monitored as per sponsors SOPs and internal audits may be conducted by the sponsor. Monitoring will include source data verification.

19. Trial committees

There will be regular meetings between the PI, and research fellow as part of study management. The safety committee will comprise of the PI and co-investigators (Barts Health NHS Trust neurologists) who will address any safety issues that may arise in the study.

20. Finance and funding

The study is funded by Merck Serono Ltd. (Bedfort Cross, Stanwell Road, Feltham, TW14 8NX, UK).

21. Indemnity

NHS indemnity scheme will apply. It provides cover for the design, management, and conduct of the study.

22. Dissemination of research findings

Patients will be asked at the time of consent whether they would like to be informed of the study outcome. If they request to be informed, they will contacted by post with a lay summary of the trial findings.

Depending on the study outcome, it may be published in a peer-reviewed scientific journal, and the conclusions disseminated through poster presentation at an international conference.

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