Worms for immune regulation of multiple sclerosis (WIRMS)

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SIGNATURE PAGE

The WIRMS Trial, Final version 1.0, dated 17 February 2011.
Written and approved by:

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Colin Ranshaw **Trial Pharmacist**	Date

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SYNOPSIS

Title	Worms for immune regulation of multiple sclerosis
Acronym	WIRMS
Short title	MS and Hookworm
Chief Investigator	Dr Cris S. Constantinescu
Objectives	Whether controlled parasitic infection with *N. americanus* (25 larvae per patient) reduces the cumulative number of gadolinium enhancing (Gd+) lesions in multiple sclerosis (MS) at month 9 in comparison to baseline and placebo.
Setting	Secondary Care
Sample size estimate	36 patients per arm (1:1 randomisation) are needed to show 70% reduction (relative risk 0.3) between m3 and m9 with 95% power (based on 2-tailed significance of 5%). 70% reduction is comparable to results of trials with IFN-beta. This is a conservative calculation because the aimed decrease is < 95% seen by Correale in 12 patients whilst infection led to a biological impact achieved by experimental infection in our hands.
Number of participants	72
Inclusion criteria	1) Relapsing remitting MS (RRMS) (McDonald criteria) and secondary MS with super imposing relapse on condition that they fulfil the next conditions, MRI scan consistent with MS by Barkhof or Fazekas criteria 2) Patients with at least 1 relapse in the last 12 months or 2 in the last 24 months; 3) Patients with Expanded disability status scale (EDSS) score in the range of 0 to 5.5 at the screening and week 0 visit 4) Patients of both genders, age >18 years and $\leq$ 65 years 5) Women of child bearing potential, (who have a negative pregnancy test) must agree to use methods of medically acceptable forms of contraception during the study. 6) Be able and willing to comply with study visits and procedures per protocol. 7) Understand and sign consent form at the screening

Exclusion criteria	No populations at risk of severe illness or death will be included in this study • Life expectancy < 6 months. • Patient is < 5 years free of malignancy, except treated basal cell skin cancer or cervical carcinoma in situ. • Patient with grade III/IV cardiac problems as defined by the New York Heart Association Criteria. (i.e., congestive heart failure, myocardial infarction within 6 months of study) • Patients with severe and/or uncontrolled medical condition. • Pregnancy, lactation or intention to become pregnant during the course of the study (please also see above under inclusion criterion 5) • Patient has a known diagnosis of human immunodeficiency virus (HIV) infection. • Anaemia (Hb <10 g/dL for females, <11 g/dL for males) • Prior or present evidence of parasitic infection; prior treatment with anti-helminthic drugs in the last 6 years. • Patient with serious medical or psychiatric illness that could potentially interfere with the completion of the study treatment according to this protocol • History of poor compliance or history of drug/alcohol abuse, or excessive alcohol consumption that would interfere with the ability to comply with the study protocol, • Severe asthma, allergy, other autoimmune disease or any condition that the physician judges could be detrimental to subjects participating in this study; including deviations deemed clinically important from normal clinical laboratory <u>Previous treatment</u> • Treatment with interferon or glatiramer acetate within 8 weeks prior to baseline or immunosuppressive drugs within 12 weeks prior to baseline • Treatment with bone marrow transplantation, total lymphoid irradiation, monoclonal antibodies (other than natalizumab, umbilical cord stem cells, AIMSPRO at any time prior to baseline • Treatment with corticosteroids or ACTH within 4 weeks prior to baseline • Treatment with any investigational agent within 12 weeks prior to baseline

Description of interventions	Controlled infection with 25 live hookworm larvae or placebo
Duration of study	Patient participation is 12 months. The study will run for 2 years.
Randomisation and blinding	Randomised double-blinded placebo controlled study.
Outcome measures	**Primary Outcome Measure:** The primary endpoint is the cumulative number of new or enlarging Gd+ lesions at month m9 **Secondary outcome measures:** • Percentage of CD4+CD25+foxp3+cells • Percent suppression in Treg assay; • Cumulative number of newly active lesions (new Gd+ T1; new and enlarging T2) at m9
Statistical methods	A Poisson regression model with allowance for possible overdispersion through estimation of robust standard errors. .

ABBREVIATIONS

AE	Adverse Event
Breg	B regulatory Cells
CI	Chief Investigator overall
CRF	Case Report Form
DSMC	Data Safety Monitoring Committee
EBV	Epstein Barr Virus
EDSS	Expanded disability status scale
EOS	End of Study
GA	Glatiramer acetate
GCP	Good Clinical Practice
IMRG	Immune Modulation Research Group
MBP	myelin basic protein
MHRA	Medicines and Healthcare products Regulatory Agency
MS	Multiple Sclerosis
MSFC	Multiple sclerosis functional composite
MS-QOL	Multiple sclerosis – Quality of Life
N. americanus	*Necator americanus*
NHS	National Health Service
NK	Natural Killer Cells
NKT	Natural Killer T cells
PAF	platelet activation factor
PBMC	Peripheral blood mononuclear cells
REC	Research Ethics Committee
R&D	Research and Development
RRMS	Relapsing remitting MS
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SUSAR	Suspected Unexpected Serious Adverse Reaction
Treg	T regulatory Cells

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SUMMARY

There is evidence that certain parasitic infections may protect against autoimmune or inflammatory diseases, including multiple sclerosis (MS), asthma and type1 diabetes. The 'hygiene hypothesis' postulates exposure to infectious agents confers protection against these disorders (1). One putative mechanism depends on the activity of regulatory T cells (Treg), naturally occurring or induced cells that prevent excessive immune activation and autoimmunity. Reports in the last 5 years lend credence to the hygiene hypothesis in MS: epidemiological investigations show an inverse relationship to infections with the nematode *Trichuris*, (2) and a study with serial clinical, immunological and MRI follow-up shows MS patients developing intestinal parasitoses have much milder disease course compared with uninfected matched MS controls followed over 5 years (3). A role for Treg and also a novel population of B regulatory (Breg) cells is suggested in this study. At the University of Nottingham we have extensive experience with human parasite research and have completed essential safety studies of controlled infection with hookworm in normal volunteers (4) and people with atopy. Asthma and Crohn's disease studies are underway and show an immunological effect even with 10 larvae. Here we propose the first controlled parasite exposure study in patients with relapsing MS with in 36 patients 25 hookworm larvae vs 36 patients with placebo. Patients will be followed clinically (relapse rate, disability scores), immunologically and radiologically (serial MRI scans with Gadolinium) for 1 year. The cumulative number of new and active lesions on T2 weighted MRI will be the primary outcome measure. Regulatory network induction (Treg induction, Breg/Tr1 and NK) will be the immunological secondary outcome measure. Relapse rate will be secondary clinical outcome measure. A number of clinical, MRI and immune parameters will be exploratory measures. Cytokine profiles, eosinophil and egg counts, IgE and IgG subsets and IgE/IgG4 ratios will be measured, to relate altered immune responses to disease modulation. Immune responses will be assessed to neuroantigen and to mitogen, and parasite antigens (excretory/secretory products). This study will be an essential early step in assessing the potential for therapeutic immunomodulation with parasites in MS.

BACKGROUND INFORMATION AND RATIONALE

Introduction and Background

The aim of the study is to determine whether controlled infection with a clinically safe number of larvae of hookworm is protective in terms of reducing MRI activity in relapsing MS and results in an immune response that induces immunoregulatory mechanisms such as activation of regulatory networks with lymphocytes (including regulatory T cells (Treg)) that suppress overactive immune responses such as occur in MS. We also plan to determine the effect of the hookworms on relapses during 1 year study.

A study of people with MS naturally infected with intestinal parasites did show significant protection over 5 years (3), and the levels of biological markers of the infection and some immune substances triggered by it were similar to the ones we obtained with controlled infection in normal volunteers, allergic and asthmatic people. We think the study has a genuine potential to benefit people with MS, and there is known interest in the MS patient community. At the therapeutic doses proposed here, this is an innocuous infection. Natural hookworm infection affects 1 billion people worldwide, often without symptoms unless the parasite load is very high (5). Only about 10% of hookworm-infected people have significant medical problems, in particular anaemia and protein deficiency. In fact, the parasite is sufficiently harmless to warrant experts' recommendation that the best way of dealing with these deficiencies in developing countries is to correct anaemia and nutritional deficiencies in

addition to deworming, as the parasite itself is only responsible for a small percentage of the multifactorially determined deficiencies (6, 7). In addition, blood loss is 10 times less severe with *N. americanus* than with the other hookworm, *Ancylostoma duodenalis*; therefore anaemia due to hookworm is considerably less common with *N. americanus*. (8)The high parasite load in endemic areas is in large part due to high rates of reinfection, which is virtually impossible in western conditions. Our controlled exposure studies have shown good tolerability and safety; the risk of infecting others and auto-infection virtually is nil in Western standard hygiene conditions. Mebendazole, used to eradicate the worm at the end of the studies, has been 100% effective in our studies. Resistance to mebendazole is largely related to reinfection and allergy to mebendazole is exceedingly rare.
Many people with MS when asked stated they would prefer an innocuous infection with microscopic larvae to a man-made product that may have more side effects. If the protective mechanisms are determined these studies may also lead to new ways of treating MS, possibly by selecting only the specific chemical components of the worms and the immune response to them that confer protection.

Rationale for the Study

The increase in MS in the Western world, along with other autoimmune inflammatory diseases and asthma may be attributed to decreased exposure to infections such as gut parasites due to improved hygiene ('the hygiene hypothesis') (1, 9). In animal models, controlled parasite infections including hookworms and related worms protect against MS-like disease (10). Parasites have evolved host-specific molecular mechanisms to dampen or condition the excessive immune responses against them and thus survive (9). These parasites induce regulatory mechanisms including Treg and a novel class of B cells that also dampen immune responses called Breg and were recently shown to improve MS in natural infection (3, 11). They may suppress a class of lymphocytes that cause most damage in MS, Th17 cells. We will produce, with controlled exposure, a similar response to those associated with protective natural exposure in MS. We have the unique combination of expertise in hookworm biology, controlled parasite exposure and immunology of MS and MS trials and our data from our other human studies indicate this is a safe and tolerable intervention of significant potential. We have state of the art imaging facilities and a track record of utilizing magnetic resonance imaging for the study of MS, including its use as outcome measure in clinical trials.

Past and Current Research

The study rationale is the inverse relation between parasite infections, particularly with nematodes, and inflammatory/autoimmune diseases including asthma and MS. The inverse relationship may explain the rise in these inflammatory diseases in the developed world, forming the basis of the updated "hygiene hypothesis" (12). The common rise in MS and asthma/allergy and the evidence that they do co-exist (13), contrary to initial reports that they are mutually exclusive (MS being Th1 and allergy Th2), suggest common mechanisms, namely an over-activity of Th17 cells (14) and a dysfunction of regulatory (Treg cells), that normally prevent or suppress autoimmunity. MS patients are defective in naturally occuring Treg that are CD4+CD25+ (or $CD25^{high}$) and express transcript regulator foxp3 (15). Treatment with glucocorticocoids up regulates Treg in MS, as we have observed (24) and asthma (16). Recent data show striking inverse relationship of MS to *Trichuris* infection across 5 continents (2). The increase in MS in the French West Indies is attributed to a significant reduction in gut parasite infection (17). A recent 5 year prospective study on 12 patients with relapsing remitting MS who became infected with gut parasites showed they had fewer relapses, less MRI activity and evidence of immune deviation and Treg increase

compared with matched MS patients without infection (3). Follow-up data of these patients shows novel mechanisms via Breg cells, protective IL-10-producing immune regulatory B cells firstly described in MS-like disease in mice (18). These findings encouraged studies of controlled infection with worms in atopy, asthma and Crohn's disease at Nottingham (4, 19), after an initial study on healthy volunteers showed the dose ranges of 10-100 larvae to be safe, associated with only transient pruritus, and to induce a biological effect that was compatible in magnitude to that reported in the Correale study of natural infection. Our expertise and the size of our MS population allow a special opportunity for an exploratory MS study in Nottingham.

DETAILS OF THE IMP

Description

Hookworm larvae (*Necator americanus*)

The Immune Modulation Research Group (IMRG) at the University of Nottingham is licensed to manufacture *Necator americanus* larvae, and the Manufacturing license number UK MIA(IMP) 3057. The larvae were manufactured according to GMP guidelines.
The containers will hold 25 actively motile hookworm larvae and will be administered once.

Placebo

The placebo vials contain pharmacopoeial grade water and are administered once.

Mebendazole

In this study mebendazole is used to eradicate the hookworm and is classed as a non-IMP. Patients will receive mebendazole chewable tablets prescribed through the hospital pharmacy. The normal brand is Janssen-Colag sold as Vermox (6 tablet pack), although non-proprietary versions may be used.
The participants take 100mg Mebendazole twice a day for 3 days.

Packaging and labelling

Packaging and labelling of the Active (Hookworm) and Placebo will be provided by Immune Modulation Research Group (IMRG) at the University of Nottingham and QP released for use in the clinical trial.
Mebendazole will be used from NUH NHS Trust Pharmacy stock.

Storage, dispensing and return

The IMP and placebo will be stored by IMRG until requested for the specific participant visit. The IMP or placebo will be transported as per the IMRG SOP. In the clinic the solution is pipetted onto a gauze pad of a sticking plaster by a qualified and experienced member of IMRG and immediately passed to the clinic nurse or investigator to administer to the participants' forearm. There are no returns.

Mebendazole is stored according the hospital pharmacy SOPs and dispensed through the hospital pharmacy using normal prescription forms. Unused Mebendazole tablets will not be collected.

Known Side Effects

Side effects related to Hookworm larvae

Skin – local irritation, pruritis.
Respiratory – cough, wheeze.
Gastrointestinal – diarrhoea, abdominal discomfort/pain/cramps, nausea, flatulence
Haematological – anaemia.

Side effects related to water for injection

none

Side effects related to Mebendazole

Mebendazole will be given at 9 months to eradicate the hookworms.
Adverse effects of mebendazole are listed below:

Frequency estimate:

Very Common:	10%
Common:	1 - <10%
Uncommon:	0.1 - <1%
Rare:	0.01 - <0.1%
Very Rare:	<0.01%

Side-effects: rarely abdominal pain, diarrhoea; hypersensitivity reactions (including exanthema, rash, urticaria, and angioedema) reported

STUDY OBJECTIVES AND PURPOSE

Purpose

The aim of the study is to determine whether controlled infection with a clinically safe number of larvae of hookworm results in an immune response that is protective in relapsing MS.

Primary Objective

Whether controlled parasitic infection with *N. americanus* (25 larvae per patient) reduces the cumulative number of gadolinium enhancing (Gd+) lesions in MS at month 9 in comparison to baseline and placebo.

Secondary Objectives

Whether controlled parasitic infection with *N. americanus* (25 larvae per patient) induces an increase in foxp3 positive ($CD4+Cd25^{high}$) T cells (Treg) in people with MS in comparison to baseline and placebo.

Whether controlled parasitic infection with *N. americanus* (25 larvae per patient) reduces relapses and disability in MS patients compared with placebo.

STUDY DESIGN

Study Configuration

Single centre, randomised, double-blind, placebo controlled study of live hookworm larvae (*N. americanus*) in patients with MS.

Primary endpoint

The primary endpoint is the cumulative number of new or enlarging Gd+ lesions at month 9

Secondary endpoints

- Percentage of cells positive simultaneously for CD4, CD25 and foxp3
- Cumulative number of newly active lesions (new Gd+ T1; new and enlarging T2) at month 9
- Change in expanded disability status scale (EDSS) at month 9.

Eradication: All subjects will receive mebendazole 100 mg bd for 3 days at month 9 (which effectively eradicates the worms) and be assessed at month 10 for eradication and persistence of an anti-hookworm immune response. Patients will be reassessed and a final MRI scan, immunological tests, physical and neurological examinations will be obtained at month 12, to rule out the theoretical possibility of an inflammatory rebound after eradication. They will be followed clinically in the MS clinic and monitored for any rebound effect following eradication.
Efficacy analysis will be performed when all patients have received treatment for 9 months, have been dewormed and have had the final MRI scan.

Randomisation and Blinding

Patients will be randomised with equal probability to the two treatment arms based on a computer generated pseudo-random code using random stratified permuted blocks of randomly varying size, created by the Nottingham Clinical Trials Unit (CTU) in accordance with their SOP and held on a secure server

The research nurses and local investigators administering the treatment, the participants, and those assessing the outcomes will be blinded to group assignment.
The statistical analysis will be partly blind as the placebo arm will be identifiable through the compliance measure (egg count).

The data monitoring committee may have access to unblinded data but will have no contact with study participants.

Maintenance of randomisation codes and procedures for breaking code

Access to the sequence of treatment assignments will be confined to the CTU Data Manager and will be concealed until interventions have all been assigned and recruitment, data collection, and all other study-related assessments are complete. Investigators will access the treatment allocation for each subject by means of a remote, internet-based system developed and maintained by the Nottingham CTU.

The code may be broken in the event of a medical emergency when further treatment is dependent on knowledge of the actual study treatment received. The web-based randomisation page has the option to unblind the treatment to authorised members of the research team.

Trial management

The study is a single centre study.
An independent data and safety monitoring committee (DSMC) will be convened and the members will be a senior neurologist, immunologist and statistician. This committee will have access to unblinded data but no formal analysis for efficacy will be performed.

Duration of study and participant involvement

The study will run for 2 years. Patient participation is 12 months.
The study will start enrolment in June 2011 and recruitment is for 12 months.

End of the study

The end of the study is the last visit of the last patient enrolled in the study.

Criteria for terminating study

We do not expect to stop the study prematurely because the work of Correale *et al.* (3), showed that the parasites are well tolerated for 5 years (the life span of *N americanus*) following natural infection with doses of parasites capable of inducing immune changes of the magnitude that have been shown in volunteers, asthma/atopy and Crohn's disease.

Trial will be stopped if more than 6 patients have SAE deemed directly related to the parasite (severe intractable diarrhoea, gastrointestinal bleeding, severe respiratory discomfort).

SELECTION AND WITHDRAWAL OF PARTICIPANTS

Recruitment

Patients will be identified, approached and recruited from our ~3000-patient MS clinic by their treating consultant. Potential participants, identified through their medical notes, will be contacted by their consultant via an invitation letter. Posters will be displayed in the clinic area and patients interested patients can speak with their consultant. There is much interest in this trial among patient groups; we receive daily inquiries. We will take referrals from other centres with whom we have longstanding collaborations. We anticipate recruiting 5-7 patients per month over 12-13 months.

Inclusion criteria

1) Relapsing remitting MS (RRMS) (McDonald criteria) and secondary MS with super imposing relapse on condition that they fulfil the next conditions, MRI scan consistent with MS by Barkhof or Fazekas criteria
2) Patients with at least 1 relapse in the last 12 months or 2 in the last 24 months;
3) Patients with Expanded disability status scale (EDSS) score in the range of 0 to 5.5 at the screening and week 0 visit
4) Patients of both genders, age >18 years and $\leq$ 65 years

5) Women of child bearing potential, (who have a negative pregnancy test) must agree to use methods of medically acceptable forms of contraception during the study.
6) Be able and willing to comply with study visits and procedures per protocol.
7) Understand and sign consent form at the screening

Exclusion criteria

No populations at risk of severe illness or death will be included in this study

- Life expectancy < 6 months.
- Patient is < 5 years free of malignancy, except treated basal cell skin cancer or cervical carcinoma in situ.
- Patient with grade III/IV cardiac problems as defined by the New York Heart Association Criteria. (i.e., congestive heart failure, myocardial infarction within 6 months of study)
- Patients with severe and/or uncontrolled medical condition.
- Pregnancy, lactation or intention to become pregnant during the course of the study (please also see above under inclusion criterion 5)
- Patient has a known diagnosis of human immunodeficiency virus (HIV) infection.
- Anaemia (Hb <10 g/dL for females, <11 g/dL for males)
- Prior or present evidence of parasitic infection; prior treatment with anti-helminthic drugs in the last 6 years.
- Patient with serious medical or psychiatric illness that could potentially interfere with the completion of the study treatment according to this protocol
- History of poor compliance or history of drug/alcohol abuse, or excessive alcohol consumption that would interfere with the ability to comply with the study protocol,
- Severe asthma, allergy, other autoimmune disease or any condition that the physician judges could be detrimental to subjects participating in this study; including deviations deemed clinically important from normal clinical laboratory

<u>Previous treatment</u>

- Treatment with interferon or glatiramer acetate within 8 weeks prior to baseline or immunosuppressive drugs within 12 weeks prior to baseline
- Treatment with bone marrow transplantation, total lymphoid irradiation, monoclonal antibodies (other than natalizumab, umbilical cord stem cells, AIMSPRO at any time prior to baseline
- Treatment with corticosteroids or ACTH within 4 weeks prior to baseline
- Treatment with any investigational agent within 12 weeks prior to baseline

Expected duration of participant participation and involvement

Study participants will be participating in the study for 52 weeks and will have to come to the Queen's Medical Centre for regular visits.

Removal of participants from therapy or assessments

Participants may be withdrawn from the study either at their own request or at the discretion of the Investigator. The participants will be made aware that this will not affect their future care. Participants will be made aware (via the information sheet and consent form) that should they withdraw the data collected to date cannot be erased and may still be used in the final analysis.

Informed consent

Potentially eligible participants or their nominated representative (other individual or other body with appropriate jurisdiction) will be asked if they are interested in taking part in this research. If so, the investigator or their nominee, e.g. from the research team or a member of the participant's usual care team will explain the details of the study and provide a participant information sheet, ensuring that the participant has at least 24 hours to consider participating or not. The investigator will answer any questions that the participant has concerning study participation and the participant may ask as many questions as needed to help decide whether or not they would like to take part

If needed, the usual hospital interpreter and translator services will be available to assist with discussion of the study, the participant information sheets, and consent forms, but the consent forms and information sheets will not be available printed in other languages. It will be explained to the potential participant that that entry into the study is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time but attempts will be made to avoid this occurrence. In the event of their withdrawal it will be explained that their data collected so far cannot be erased and we will seek consent to use the data in the final analyses where appropriate.

All participants must provide written evidence of informed consent by means of a form to be signed and dated by the participant before they enter the study, defined as undergoing any study-related interventions (including physical examination and history taking).

One copy of the consent form will be kept by the participant, one will be kept by the investigator, and a third will be retained in the patient's hospital records.

With the participant's permission, their GP will be informed of their participation in the study.

Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the study, continuing consent will be obtained using an amended consent form which will be signed by the participant.

STUDY ASSESSMENTS

A summary of assessments at each time point is given in the schedule below

Study Schedule

Month	**-¼**	**0**	**¼**	**½**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**12**
Visit	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Informed consent	x														
Incl/excl criteria	x														
Medical history	x														
Concomitant medication	x		x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination	x						x			x			x	x	
Vital signs	x			x	x	x	x	x	x	x	x	x	x	x	
ECG	x														
Clinical Chemistry: LFT, UA	x														
Haematology: FBC	x				x		x			x			x		x
MRI							x	x	x	x	x	x	x		x
Urine Pregnancy test	x				x	x	x	x	x	x	x	x	x		x
Randomisation to Hookworm v placebo		x													
EDSS	x				x	x	x	x	x	x	x	x	x	x	
MSFC	x												x		x
MSQoL or MuSIQol, FSS, SF-36 and MSIS29	x												x		
Adverse events			x	x	x	x	x	x	x	x	x	x	x	x	x
Telephone check			x												
Immunology: routine Ig assays,	x				x		x		x		x		x	x	
Stool egg count	x				x		x		x		x		x	x	x
Treg (x) Treg assays (xx)	x, xx			x	x	x	x	x		x			x, xx	x	
Immunology research assays	x									x			x		x
Mebendazole													x		

Screening visit (visit 1, month -¼)

The screening visit will take place over 1 week to make sure that the patient is eligible to be randomised. At the screening visit (month -¼) patient's inclusion and exclusion criteria will be assessed and if the patient is eligible they will sign a consent form.

Blood samples will be taken at this visit for routine blood tests (FBC, LFT, UA ,GFR), immunology, Tcell measurements and Treg marker studies (CD4+, DC25+, FOXP3+ cell counts and Treg functional assays based on proliferation assays).
The patient will be asked for a stool sample to check for parasite eggs and gut flora. A medical history will be obtained and concomitant medication will be recorded. Patients will have a general physical and a neurological examination using the expanded disability status scale and multiple sclerosis functional composite (EDSS and MSFC); vital signs recorded and will undergo an ECG.
Female patients of child bearing potential will be asked to do a urine pregnancy test.
The patients will also be asked to complete several questionnaires at this visit. The questionnaires are the Fatigue Severity Score (FSS), a MS related quality of life questionnaire (MSQol or MuSIQoL) and MSIS29 (Multiple sclerosis impact scale).

Baseline visit and randomisation (Visit 2. Month 0)

At baseline visit (visit 2, month 0), patients will be assessed for interim changes since the screening visit.
Randomisation will then be to receive either 25 larvae of *N americanus* or placebo (an equal volume of water). The IMP solution is pipetted onto a gauze pad of a sticking plaster by a qualified and experienced member of IMRG and immediately passed to the clinic nurse or investigator who will place it over the participants' volar surface of the non-dominant forearm (or more affected forearm if they have upper MS-related limb dysfunction) for 24 hours.

36 patients will receive hookworm larvae and 36 patients will receive placebo.
In preparation for infection of study subjects the solution is pipetted onto a plaster dressing which is then placed on the upper forearm for 24 h. After 24h the patient removes the plaster and places it in a container with 70 % alcohol. The patient returns the container to the hospital at the next follow-up visit.The container is transported back to the IMRG lab where it can be examined microscopically to ensure all the larvae left the dressing.
Participants will be contacted by telephone after 1 week to monitor for side effects.

Follow-up visits (visit 4-14, month ½-10)

The patients need to visit the clinic at the regular times after infection. The assessments are according to the Study Schedule.

In summary the assessments at follow-up visits are as follows:
Concomitant medication, adverse events and the symptom diary are checked and recorded at each visit. Vital signs are recorded at visit 4- 14 and a physical examination is conducted at visits 7, 10, 13 and 14.
Female patient of child bearing potential will undergo a urine pregnancy test at visits 5-13 and a blood sample for full blood count will be taken at visits 5, 7, 10 and 13.

Routine immunology blood sample taken at visit 5, 7, 9, 11, 13, 14. Further blood samples are taken for specialised immunology tests which could include but are not restricted to Tcell measurements (these will include Th1/Th2/Th7 cytokine assays), Treg marker studies (these include CD4+, DC25+, FOXP3+ cell counts and Treg functional assays based on proliferation assays). These are done at 4-8, 10, 13 and 14.

The participants will also bring in a stool samples to be assessed for the presence of hookworm eggs and saved for gut flora studies at visits 5, 7, 9, 11, 13 and 14.

Regular MRI scans in 1.5 T scanners will be conducted using the University of Nottingham scanners in the Medical School. In total there will be 8 scans, one run-in scan at 3 months (visit 7) and then monthly scans at 4, 5, 6, 7, 8 and 9 months (visits 8-13). As the most appropriate primary outcome measure in a phase II study is the cumulative number of Gd+ lesions, serial scans are needed to obtain these numbers. This design also ensures a reduction in the sample size needed.

The patient is assessed with EDSS at monthly intervals at visits 5-14 and with MSFC at visit 13. The questionnaires MSQol or MuSIQol, FSS and MSIS29 are only done at visit 13.

All patients will be treated with mebendazole 100 mg bd for 3 days at 9 months (visit 13) and assessed at the end of the trial (visit 15, 12 months) for eradication and persistence of an anti-hookworm immune response. They will be followed clinically in the MS clinic and monitored for any rebound effect following eradication.

If patients leave the study early they will come to the clinic to receive the eradication treatment and follow-up assessments for the persistence of an anti-hookworm immune response.

Final visit or early termination visit

The final visit at 12 months (visit 15) will record concomitant medication and any adverse events. Patient will undergo their final MRI scan and give a blood sample for FBC and specialised research tests. The participants will also bring in a stool samples to be assessed for the presence of hookworm eggs and saved for gut flora studies The patient is assessed using MSFC and the questionnaires MSQol or MuSIQol, FSS and MSIS29 will have to be completed. Female patients of child bearing potential will undergo a urine pregnancy test.

Clinical assessments, unscheduled visits, relapse treatment

Neurologic assessment will be performed by the research team's examining doctor. Symptoms and adverse events will be monitored by a treating physician. Relapses are defined as new or recurrent neurologic symptoms not associated with fever or infection lasting >24h and accompanied by objective signs on examination. Subjects will notify study coordinators (V Orpe or M Harrison of our MS Trials Unit) within 72h of onset and be evaluated by the examining doctor within 120h of onset. If the treating doctor finds that the patient needs treatment for relapse, this may only be methylprednisolone 2500-3000 mg over 3-5 days. MRI will be on schedule unless subject received steroids in the 7 days preceding the MRI; in this case MRI is omitted at that visit.

Sample handling, storage and disposal

The blood samples are analysed and stored in Prof Constantinescu's laboratory. The blood samples collected for routine blood tests will be analysed immediately after collection.
Fresh immunological analyses will be done on part of the blood samples [No more than 50 ml blood first and last visit, no more than 30 for the other visits] in the labs of Profs Constantinescu and Pritchard.
qPCR will be done on blood samples in Profs Constantinescu's lab.
The blood samples collected for Tcell measurements will be stored and analysed together with the samples collected at the other time points

The stool samples are transported in suitable containers to Prof Pritchard's laboratory, analysed, and stored there. Only Prof. Constantinescu or Prof. Pritchard or designated researchers working under their supervision will have access to the samples.

The samples will be identified by the study name participant number and date.

Analysed samples will be destroyed (discarded as biological waste). Residual samples will, with the participant consent, be stored for future studies.
The samples will be stored under the University of Nottingham Human Tissue License 12265 and in accordance with the Human Tissue Act 2004, once ethical approval covering the samples expires. These samples will be coded for study use, but the link between the patient and the code will be deleted.

MRI analysis

MRI analyses will be performed centrally in Prof. Auer's group. Dr Chris Tench of the team of the CI, a physicist with expertise in image analysis and advanced statistics, will assist and guide a blinded analysis of the MRI data.

Compliance

Patients will need to visit the hospital for various assessments 14 times over the space of a year.

Accountability for drugs & placebos

The IMP is manufactured by IMRG and handled by a member of their staff until it is transferred onto the sticking plaster. The used vials are taken back to IMRG and disposed according to their SOP.

Unused Mebendazole tablets will not be collected.

Concomitant medication

Concomitant medications are all prescribed medications (drugs) being taken regularly by a participant on entry to the trial, and all medication prescribed on a regular basis in addition to the trial treatment during the trial.

All concomitant medications and treatments must be documented (using generic name and trade name as appropriate) and also in the participant's medical records. Include any changes to these treatments and dosage.

NB: Concomitant medications present at baseline and which do not interfere with the assessments should be kept constant as much as possible from screening throughout the trial to treat the itch that may occur with hookworm infection (the rash would need no treatment per se unless it itches).

Concomitant medications/treatment should be kept to a minimum during the trial. However, if considered necessary for the participant's welfare and unlikely to interfere with the trial drug, they may be given at the discretion of the investigator according to the local standard of care.

Participants will be advised that they can take over-the-counter anti-histamine tablets to treat the itch and rash that may result from the hookworm infection.

STATISTICS

Estimation of Sample Size.

This is based on the data presented in table 2 of Tubridy et al (23) which indicated that the mean number of new lesions per patient over 6 months was 8.8 with SD = 11.2. 36 patients per arm (1:1 randomisation) are needed to show 70% reduction (relative risk 0.3) between m3 and m9 with 95% power (based on 2-tailed significance of 5%).

A 70% reduction is comparable to results of trials with IFN-β and is a conservative calculation because the aimed decrease is < 95% of that seen by Correale in 12 patients.

Outline of Statistical methods

Statistical analyses will be performed using the current version of Stata.

These can be summarised as:

- Description of subject characteristics (including participant disposition & baseline assessments)
- Assessment of study quality (including losses to follow-up and compliance)
- Analysis of efficacy
- Analysis of safety

For descriptive analyses continuous data will be summarised in terms of the mean, standard deviation, median, lower & upper quartiles, minimum, maximum and number of observations. Categorical data will be summarised in terms of frequency counts and percentages. The proportion of subjects with missing values will also be given for each variable.

The primary efficacy parameter will be the relative risk between the two treatment arms of the number of new lesions by month 9. This will be estimated by a Poisson regression model with allowance for possible overdispersion through estimation of robust standard errors and results will be presented in terms of a point estimate of the relative risk together with the associated 95% confidence interval and two-sided significance level.

The model will include terms to adjust for covariates strongly associated with outcome, to be specified in advance in the Analysis Plan.

No adjustments to significance levels will be made for multiplicity.

In accordance with the “intention to treat” (ITT) principle, efficacy for both primary and secondary outcomes will be assessed on the full analysis set (see “Definition of populations analysed” below) with use of the per protocol set as a sensitivity analysis.

Safety analyses will be performed on the safety set.

Full details will be provided in a Statistical Analysis Plan, further details of which will be supplied later in a separate document to be finalized before unblinding.

Definition of populations analysed

The Analysis populations are defined as:

- Safety set: All randomised participants who receive one dose of the study treatment.
- Full Analysis set: All randomised participants, who are dispensed one dose of study treatment and for whom at least one post-baseline assessment of the primary endpoint is available, either directly or indirectly (e.g. by multiple imputation).
- Per protocol set: All participants in the Full Analysis set who are deemed to have no major protocol violations that could interfere with the objectives of the study.

Procedures for missing data

Missing covariate and response values will be handled by multiple imputation separately by treatment arm. In particular, the imputation for missing response data at month 9 will incorporate information on earlier response data and other variables thought likely to account for the missing data.

Estimated parameter values will be combined using Rubin's rules.

A sensitivity analysis in which missing outcome data are assumed to be missing not at random will also be performed for the primary outcome and for response. This may be accomplished for example by replacing the imputed primary response values by the observed values least favourable to the active treatment and re-estimating the treatment effect.

ADVERSE EVENTS

Definitions

An adverse event is any unfavourable and unintended sign, symptom, syndrome or illness that develops or worsens during the period of observation in the study.
An AE does include a / an:

1. exacerbation of a pre-existing illness.

2. increase in frequency or intensity of a pre-existing episodic event or condition.

3. condition detected or diagnosed after medicinal product administration even though it may have been present prior to the start of the study.

4. continuous persistent disease or symptoms present at baseline that worsen following the start of the study.

An AE does not include a / an:

1. medical or surgical procedure (e.g., surgery, endoscopy, tooth extraction, transfusion); but the condition that lead to the procedure is an AE.

2. pre-existing disease or conditions present or detected at the start of the study that did not worsen.

3. situations where an untoward medical occurrence has not occurred (e.g., hospitalisations for cosmetic elective surgery, social and / or convenience admissions).

4. disease or disorder being studied or sign or symptom associated with the disease or disorder unless more severe than expected for the participant's condition.

5. overdose of concurrent medication without any signs or symptoms.

A Serious Adverse Event (SAE) is any adverse event occurring following study mandated procedures, having received the IMP or placebo that results in any of the following outcomes:

1. Death

2. A life-threatening adverse event

3. Inpatient hospitalisation or prolongation of existing hospitalisation

4. A disability / incapacity

5. A congenital anomaly in the offspring of a participant

Important medical events that may not result in death, be life-threatening, or require hospitalisation may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the patient or participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

MS-specific events will not be classified as SAEs unless there is an increase exacerbation of these symptoms. These symptoms are visual disturbance, dizziness, ataxia, sphincter disturbance typical of MS (overactive bladder, constipation, incontinence), paresthesia, numbness, weakness.

All adverse events will be assessed for seriousness, expectedness and causality:

A distinction is drawn between serious and severe AEs. Severity is a measure of intensity whereas seriousness is defined using the criteria above. Hence, a severe AE need not necessarily be serious.

Causality

Not related or improbable: a clinical event including laboratory test abnormality with temporal relationship to study treatment administration which makes a causal relationship incompatible or for which other drugs, chemicals or disease provide a plausible explanation**.** This will be counted as “unrelated” for notification purposes.

Possible: a clinical event, including laboratory test abnormality, with temporal relationship to study treatment administration which makes a causal relationship a reasonable possibility, but which could also be explained by other drugs, chemicals or concurrent disease. This will be counted as “related” for notification purposes.

Probable: a clinical event, including laboratory test abnormality, with temporal relationship to study treatment administration which makes a causal relationship a reasonable possibility, and is unlikely to be due to other drugs, chemicals or concurrent disease. This will be counted as “related” for notification purposes.

Definite: a clinical event, including laboratory test abnormality, with temporal relationship to study treatment administration which makes a causal relationship a reasonable possibility, and which can definitely not be attributed to other causes. This will be counted as “related” for notification purposes.

An AE whose causal relationship to the study IMP is assessed by the Chief Investigator as "possible", "probable", or "definite" is an Adverse Drug Reaction.

With regard to the criteria above, medical and scientific judgment shall be used in deciding whether prompt reporting is appropriate in that situation.

Reporting of adverse events

Participants will be asked to contact the study site immediately in the event of any serious adverse event. All adverse events will be recorded and closely monitored until resolution, stabilisation, or until it has been shown that the study medication or treatment is not the cause. The Chief Investigator shall be informed immediately of any serious adverse events and shall determine seriousness and causality in conjunction with any treating medical practitioners.

In the event of a pregnancy occurring in a study participant or the partner of a study participant monitoring shall occur during the pregnancy and after delivery to ascertain any study related adverse events in the mother or the offspring. Where it is the partner of a study participant consent will be obtained for this observation from both the partner and her medical practitioner.
All serious adverse events will be recorded and reported to the MHRA and REC as part of the annual reports. SUSARs will be reported within the statutory timeframes to the MHRA and REC as stated below. The Chief Investigator shall be responsible for all adverse event reporting.

Susars

A serious adverse event that is sudden in its onset, unexpected in its severity and seriousness or not a known side effect of the IMP and related or suspected to be related to the IMP is classed as Suspected Unexpected Serious Adverse Reaction and requires expedited reporting as per the clinical studies regulations.

All serious adverse events that fall or are suspected to fall within these criteria shall be treated as a SUSAR until deemed otherwise.

The event shall be reported immediately of knowledge of its occurrence to the Chief Investigator.

The Chief Investigator will:

- Assess the event for seriousness, expectedness and relatedness to the study IMP
- Take appropriate medical action, which may include halting the study and inform the Sponsor of such action
- If the event is deemed a SUSAR, shall, within seven days, enter the required data on the MHRA's eSUSAR website.
- Shall inform the REC using the reporting form found on the NRES web page within 7 days of knowledge of the event
- Shall, within a further eight days send any follow-up information and reports to the MHRA and REC.
- Make any amendments as required to the study protocol and inform the ethics and regulatory authorities as required

Trial Treatment Related SAEs

A serious adverse event that is unexpected in its severity and seriousness *and* deemed directly related to or suspected to be related to the trial treatment but not the IMP shall be reported to the ethics committee that gave a favourable opinion as stated below.

The event shall be reported immediately of knowledge of its occurrence to the Chief Investigator.

The Chief Investigator will:

- Assess the event for seriousness, expectedness and relatedness to the trial treatment.
- Take appropriate medical action, which may include halting the trial and inform the Sponsor of such action.
- If the event is deemed related to the trial treatment shall inform the REC using the reporting form found on the NRES web page within 7 days of knowledge of the event.
- Shall, within a further eight days send any follow-up information and reports to the REC.
- Make any amendments as required to the study protocol and inform the REC as required

Participant removal from the study due to adverse events

Any participant who experiences an adverse event may be withdrawn from the study at the discretion of the Investigator.

ETHICAL AND REGULATORY ASPECTS

Ethics Committee and Regulatory Approvals

The study will not be initiated before the protocol, informed consent forms and participant and GP information sheets have received approval from the Medicines and Healthcare products Regulatory Agency (MHRA), Research Ethics Committee (REC), and the respective National Health Service (NHS) Research & Development (R&D) department. Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted until the amendment and revised informed consent forms and participant and GP information sheets (if appropriate) have been reviewed and received approval from the REC and R&D departments. A protocol amendment intended to eliminate an apparent immediate hazard to participants may be implemented immediately providing that the MHRA, R&D and REC are notified as soon as possible and an approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC will be informed.

The study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996; the principles of Good Clinical Practice and in accordance with the Medicines for Human Use Regulations, Statutory Instrument 2004, 1031 and its subsequent amendments.

Informed Consent and participant Information

The process for obtaining participant informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The investigator or their nominee and the participant shall both sign and date the Informed Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Trial Master File. A second copy will be filed in the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the study.

The decision regarding participation in the study is entirely voluntary. The investigator or their nominee shall emphasize to them that consent regarding study participation may be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No study-specific interventions will be done before informed consent has been obtained.

The investigator will inform the participant of any relevant information that becomes available during the course of the study, and will discuss with them, whether they wish to continue with the study. If applicable they will be asked to sign revised consent forms.

If the Informed Consent Form is amended during the study, the investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Informed Consent Form by the REC and use of the amended form (including for ongoing participants).

Records

Drug accountability

The Hookworm and placebo supply will be kept in a secure, limited access storage area under the storage conditions specified by the Laboratory of Prof Pritchard.
Mebendazole is stored according the hospital pharmacy SOPs and dispensed through the hospital pharmacy.

The Investigator and Prof Pritchard's laboratory shall maintain records of the study drug's distribution to each participant. The hospital pharmacist shall maintain records of Mebendazole tablets distribution to each participant. Unused Mebendazole tablets will not be collected.

These records will include dates, quantities received, batch numbers, expiration dates, and the unique code numbers (patient trial number) assigned to the trial participant. Investigators, the laboratory of Prof Pritchard and hospital pharmacist will maintain records that document adequately that the participants were provided with the correct study medication. These records will be part of each patient's Case Report Form (CRF).

Case Report Forms

Each participant will be assigned a study identity code number, allocated at screening, for use on CRFs other study documents and the electronic database. The documents and database will also use their initials and date of birth.
CRFs will be treated as confidential documents and held securely in accordance with regulations. The investigator will make a separate confidential record of the participant's

name, date of birth, local hospital number or NHS number, and Participant Study Number (the Study Recruitment Log), to permit identification of all participants enrolled in the study in accordance with regulatory requirements and follow-up as required.
CRFs shall be restricted to those personnel approved by the Chief or local Principal Investigator and recorded on the 'Study Delegation Log.'

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.
The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded in the CRF.

Source documents

Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms, current medical records, laboratory results and pharmacy records. A CRF may also completely serve as its own source data. Only study staff as listed on the Delegation Log shall have access to study documentation other that the regulatory requirements listed below.

Direct access to source data / documents

The CRF and all source documents, including progress notes and copies of laboratory and medical test results shall made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities (e.g. MHRA, REC).

Data Protection

All study staff and investigators will endeavour to protect the rights of the study's participants to privacy and informed consent, and will adhere to the Data Protection Act, 1998. The CRF will only collect the minimum required information for the purposes of the study. CRFs will be held securely, in a locked room, or locked cupboard or cabinet. Access to the information will be limited to the study staff and investigators and relevant regulatory authorities (see above). Computer held data including the study data base will be held securely and password protected. All data will be stored on a secure dedicated web server. Access will be restricted by user identifiers and passwords (encrypted using a one way encryption method). Information about the study in the participant's medical records will be treated confidentially in the same way as all other confidential medical information.

Electronic data will be backed up every 24 hours to both local and remote media in encrypted format.

QUALITY ASSURANCE AND AUDIT

Insurance and Indemnity

Insurance and indemnity for study participants and study staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but study participants may have recourse through the NHS complaints procedures.

The University of Nottingham has taken out an insurance policy to provide indemnity in the event of a successful litigious claim for proven non-negligent harm.

Study Conduct

Study conduct will be subject to audit of the Study Master File for inclusion of essential documents; permissions to conduct the study; Study Delegation Log; CVs of study staff and training received; local document control procedures; consent procedures and recruitment logs; adherence to procedures defined in the protocol (e.g. inclusion / exclusion criteria, correct randomisation, timeliness of visits); adverse event recording and reporting; drug accountability, pharmacy records and equipment calibration logs.
The Study Coordinator, or where required, a nominated designee of the Sponsor, shall carry out a site audit every six months and an audit report shall be made to the Study Steering Committee.

Study Data

Audit of study data shall include confirmation of informed consent; source data verification; data storage and data transfer procedures; local quality control checks and procedures, back-up and disaster recovery of any local databases and validation of data manipulation. The Study Coordinator, or where required, a nominated designee of the Sponsor, shall carry out monitoring of study data as an ongoing activity.

Entries on CRFs will be verified by inspection against the source data. A sample of CRFs (as per trial risk assessment) will be checked on a regular basis for verification of all entries made. In addition the subsequent capture of the data on the study database will be checked. Where corrections are required these will carry a full audit trail and justification.

Record Retention and Archiving

In compliance with the ICH/GCP guidelines, regulations and in accordance with the University of Nottingham Code of Research Conduct and Research Ethics, the Chief or local Principal Investigator will maintain all records and documents regarding the conduct of the study. These will be retained for at least 7 years or for longer if required. If the responsible investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.
The Study Master File and study documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all study databases and associated meta-data encryption codes.

Discontinuation of the Study by the Sponsor

The Sponsor reserves the right to discontinue this study at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons. The Sponsor shall take advice from the Data Safety Monitoring Committee as appropriate in making this decision.

Statement of Confidentiality

Individual participant medical information obtained as a result of this study are considered confidential and disclosure to third parties is prohibited with the exceptions noted above. Participant confidentiality will be further ensured by utilising identification code numbers to correspond to treatment data in the computer files.

Such medical information may be given to the participant's medical team and all appropriate medical personnel responsible for the participant's welfare.

Data generated as a result of this study will be available for inspection on request by the participating physicians, the University of Nottingham representatives, the REC, local R&D Departments and the regulatory authorities.

PUBLICATION AND DISSEMINATION POLICY

The study results will be presented at scientific meetings and submitted for publication in peer reviewed journals. The participants will not be identified in any publications. The tentative time frame is as follows: Months 0-3: Study set-up, search patient database for potential candidate participants. Months 4-12: start recruitment and start study; Month 12 (end of recruitment); continue study; Month 24: end of study-last patient out; Months 24-36: data analysis.

STUDY FINANCES

Funding source

This study is funded by the MS Society

Participant stipends and payments

Participants will not be paid to participate in the study. Reasonable expenses for travel not to exceed £30 per visit per patient will be reimbursed.

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APPENDIX

Exploratory endpoints for clinical, immunological and MRI mapping

Response assessment:

- The annualized relapse rate;
- Number of DWI positive lesions (a sensitive indicator of plaque cellularity);
- Ventricular volume and cervical spinal cord area

Quality of life/patient centred measures:
Measured at baseline (month 0) and visit 12 (month 9)

- MSQoL or MuSIQol,
- SF36, FSS
- MSIS29

Safety measures:

- Use of steroids;
- Hospitalizations needed

Clinical and biological parameters to be monitored:

- Eosinophil count;
- Stool egg count;
- Percentage of CD4+CD25+foxp3+ T cells.
- PBMC/PBL are stimulated with: mitogen, superantigen, neuroantigen (whole MBP, PLP immunodominant peptide,
- Glatiramer acetate (GA);
- using PPD/TT and KLH as positive and negative controls, respectively);
- LPS/LTA, and parasite antigens (hookworm excretory-secretory products) for the most relevant markers and cytokines.
- Most MS patients' Tcells respond to whole myelin basic protein (MBP) (22).
- Proliferation will be assessed by tritiated thymidine incorporation.
- Cytokine/chemokine quantification using Luminex (available in Prof Pritchard's group at the Pharmacy School)/ cytometric bead arrays (routine use in Prof Constantinescu's lab), intracellular staining (this includes Th1/2/3/17 cytokines);
- histamine and platelet activation factor (PAF);
- qPCR or Western blot or intracellular/surface FACS for T effector/Treg-associated genes which may include:Foxp3, GITR, CTLA-4, CD31, CD103, CD62, GCR, GATA-3, t-bet, STAT1-4, smad3,4,7; PAF-R, CCR4/6, apoptosis-related genes; also NK, NKT (CD3+56+) and HLA-G+ cells (the markers we are most interested in are underlined)
- At month 0 and month 9 we will perform in vitro functional assays for Treg cells assessing the effect of CD4+25+ Treg on proliferation (to antiCD3/CD28 by 3H thymidine uptake, as developed in Prof Constantinescu's lab) of CD4+25- T effector cells (1:1 and ⅛:1 Treg:Teff). We will measure sCD23 and CD20 including $CD20^{+}IL\text{-}10^{+}$ regulatory B cells; and CD21 (receptor for sCD23 and for Epstein Barr Virus (EBV), to test if increased sCD23 as shown during helminthiasis reduces EBV binding sites).
- Treg and signature Th1/2/17 cytokines will be assessed twice at baseline 1 week apart. The preliminary results of the primary and secondary immunological endpoints

will inform these more detailed studies and dictate the extent and specifics of these studies.

- Percent suppression in Treg assay.