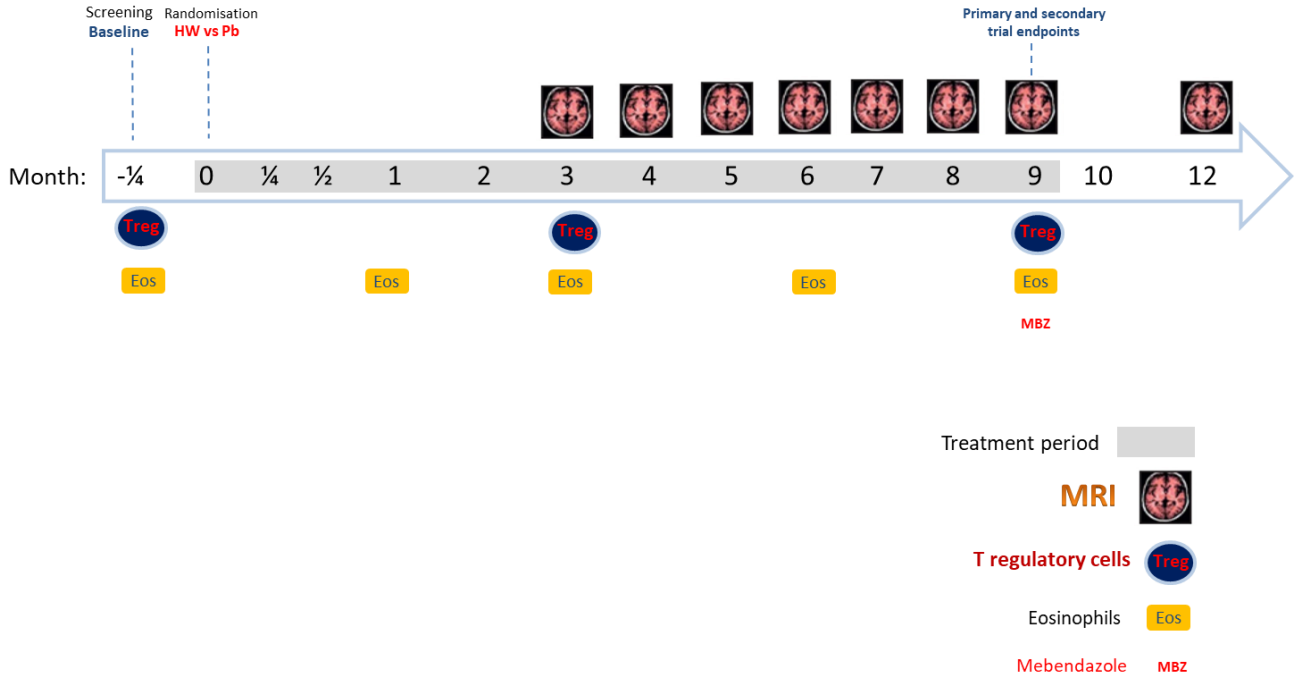
Supplementary Online Content

Tanasescu R, Tench CR, Constantinescu CS, et al. Hoowkworm treatment for relapsing multiple sclerosis: a randomized double-blinded placebo-controlled trial. *JAMA Neurol*. Published online June 15, 2020. doi:10.1001/jamaneurol.2020.1118

This supplementary material has been provided by the authors to give readers additional einformation about their work.

eAppendix 1 - Study overview.

A timeline of study visits (unit: months) and primary (MRI) and secondary (Treg) outcome measure endpoints is displayed. Participants were treated between months 0 and month 9 (grey strip). At month 9 post-infection, treatment for deworming was offered. MRIs were performed between monthly between months 3 and 9, and 3 months after.



Appendix 2 - 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: AST, ALT, urea, creatinine	x														
Haematology: Full blood count	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
CD4+CD25+foxp3+cells CD4+CD25^{high}CD127^{neg}	x, xx			x	x	x	x	x		x			x, xx	x	
Immunoglobulins	x									x			x		x
Mebendazole													x		

eAppendix 3 - Number of larvae choice, manufacturing, storage and molecular confirmation of *Necator americanus* infection

Number of larvae choice, manufacturing and storage of N americanus larvae

The number of larvae chosen was based on our previous dose-ranging studies (the number of larvae that yield ≥50 eggs/g faeces, the amount of eggs associated with reduced risk of wheeze in a nested case-control study).[1] The larvae were manufactured according to GMP guidelines by the Immune Modulation Research Group (IMRG) at the University of Nottingham (Manufacturing license number: UK MIA (IMP) 3057). Packaging and labelling of the active treatment (HW) and placebo was provided by IMRG and Qualified Person (QP)- released for use in the trial. The HW and placebo were stored by IMRG until treatment day.

Molecular confirmation of *Necator americanus* infection

To confirm the successful infection of HW cohort molecular approaches were applied. Genomic DNA was extracted directly from each faecal sample, as well as from ten negative (no-DNA template) controls, using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), according to manufacturers' instructions. For each patient the latest available faecal sample prior to anthelmintic treatment (ideally timepoint 9 and no earlier than timepoint 3) was selected for PCR-guided *N. americanus* DNA detection. Briefly, the second internal spacer region (ITS2) region was PCR-amplified using 2.5 µl of the previously described NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTATTAGTTTCTTTTCCTCCGCT-3') primers[2], 25 µl of the NEB OneTaq Mix (New England Biolabs), 22 µl of H_2O, and 2.5 µl of DNA template per reaction. Subsequently, the following thermocycling protocol was employed: 1 min at 95°C, 30 cycles of 15 s at 95°C – 15 s at 55°C – 15 s at 72°C, and a final elongation of 10 min at 72°C.

Additionally, a more sensitive real-time PCR protocol was used on faecal samples from patients for which the standard PCR protocol was not able to detect worm DNA. These assays were conducted in 7 µl volumes, containing 3.5 µl of 2X TaqMan Fast Universal PCR Master Mix (Life Technologies), 125 nmol of the previously published *N. americanus* probe (forward: 5'- CCAGAATCGCCACAAATTGTAT-3'; reverse: 5'-GGGTTTGAGGCTTATCATAAAGAA-3')[3], and 2 µl of template DNA at a concentration of 1 ng/µl. Cycling conditions consisted of an initial 2 min incubation step at 50°C, a 10 min incubation at 95°C, and 40 cycles of 95°C for 15 sec for denaturation, followed by 1 min at 59°C for annealing and extension. All reactions were conducted using the ABI 7500 Fast Real Time PCR System (Applied Biosystems™).

References

1. Scrivener S, Yemaneberhan H, Zebenigus M, et al. Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study. *Lancet*. 2001;**358(9292)**:1493-9.
2. Gasser RB, Chilton NB, Hoste H, Beveridge I. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Res*. 1993;**21(10)**:2525-6.
3. Pilotte N, Papaiakovou M, Grant JR, Bierwert LA, Llewellyn S, McCarthy JS, et al. Improved PCR-Based Detection of Soil Transmitted Helminth Infections Using a Next-Generation Sequencing Approach to Assay Design. *PLoS Negl Trop Dis*. 2016;**10(3)**:e0004578.

eAppendix 4 - MRI protocol for WIRMS trial

MRI was performed on 1.5T GE Signa HDx at QMC, University of Nottingham.

MRI images were read by a trained person in blinded fashion.

Acquired images were: 3D axial T1 weighted fast spoiled gradient echo (1×1×1mm isotropic, 256×256×156 matrix), post gadolinium (Gadovist, standard dose; Gd) acquisition of axial T2 weighted fast spin echo, and axial T2 weighted fluid attenuated inversion recovery images (0.98×0.98×3mm, matrix 256×256×60) which achieved about 13 minutes' delay between Gd injection and post Gd axial T1 weighted spin echo.

The run-in scan was performed at month 3 followed by 6 monthly MRI scans (months 4-9), and finally at month 12. T1-weighted (including Gd), T2/FLAIR/proton density-weighted, diffusion-weighted, and MP-RAGE sequences were obtained.

The choice for MRI baseline scan at month 3 was based on the HW lifecycle and the anticipated timing of development of immune regulatory mechanisms.[1]

Reference

1. Hotez PJ, Brooker S, Bethony JM, et al. Hookworm infection. *N Engl J Med*. 2004;**351(8)**:799-807.

eAppendix 5 - Treg cells measurement

Blood samples were taken at the specified visit time points in the study protocol (screening, month 1, 2, 3, 4, 6, 9 and 10 months after infection/placebo).

Peripheral blood mononuclear cells (PBMCs) from patients were isolated by a density centrifugation standard protocol using Histopaque (Sigma-Aldrich).

PBMCs were frozen at a density of 5-10 x 106 cell/mL using cryoprotective freezing medium (90 % Fetal Bovine Serum (FBS) + 10% Dimethyl sulfoxide) until completion of sample collection.

Then, PBMCs from screening and 9-month sample visits were thawed, washed and cultured overnight in RPMI 1640 with 10% FBS, 100 U/mL penicillin, 1 mg/mL streptomycin and 20 mM L-glutamine (all from Sigma-Aldrich). After this, cells were washed two times with FACS buffer (PBS + 2% FBS) and incubated with Live/Dead IR stain fluorescence (Invitrogen) under dark conditions for 30 min. Then, washed cells were staining for extracellular markers CD4, CD25 and CD127 by standard procedures (all antibodies were from BD Biosciences). For FoxP3 marker, intracellular staining was performed using the Human FoxP3 buffer set (from BD Biosciences) according manufacturer's instructions.

Isotype controls for each antibody were included and 'fluorescence minus one' samples were used to set the gating. Cells were analysed by BD FACS Canto II and Kalusa software.

Percentages of Treg as CD4+CD25++CD127- and CD4+CD25++FoxP3+ were analysed at screening and 9-month visits in infected and placebo groups.

eAppendix 6 – New T1 enhancing MRI lesions during the trial

The number of newly-enhancing-T1-weighted lesions (Figure 1a) and the number of patients with newly-enhancing-T1-weighted lesions (Figure 1b) in the hookworm (HW) (n=32) and placebo (n=34) groups during the trial. m4 is first MRI after baseline MRI. m9 is MRI for primary endpoint.

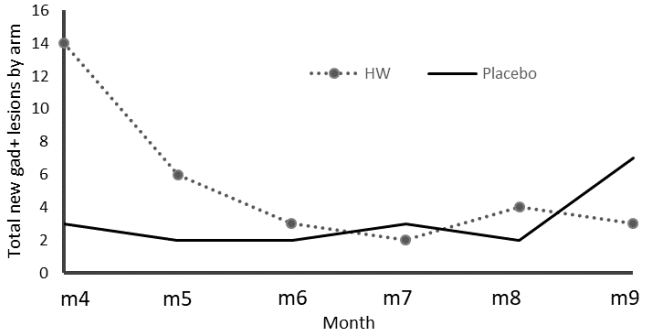


Figure 1a

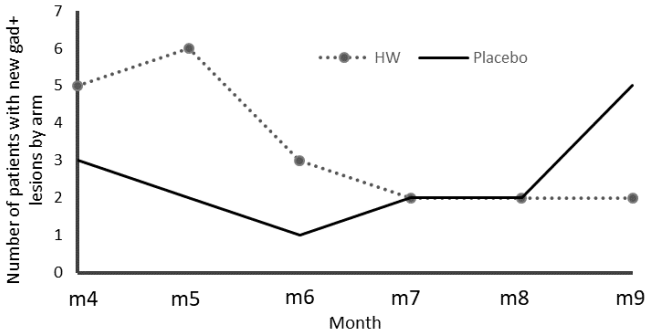


Figure 1b