Human Herpes Virus Antibody Levels in Helminth Treated and Placebo Controlled Multiple Sclerosis Patients

Peter Alan Christopher Maple

Thesis submitted to the University of Nottingham
For the degree of Master of Research
September 2017

Faculty of Medicine and Health Sciences
School of Clinical Sciences
Section of Clinical Neurology
Division of Clinical Neuroscience

DECLARATION OF AUTHENTICITY

I hereby declare that this thesis is my original work and has not been submitted for a degree or examination to any other university. I have been solely responsible for the experimental results presented, except where otherwise stated.

I confirm that where other sources of information have been used or quoted, this has been indicated and accordingly acknowledged by complete references.

Peter Alan Christopher Maple

ABSTRACT

In the UK, multiple sclerosis (MS) is the leading cause of non-traumatic disability in young adults. MS most commonly presents in a relapsing-remitting form (RRMS); however, over time, a progressive, neurodegenerative pathology dominates, and the disease enters a secondary progressive phase. Approximately, 10-15% of MS cases are primary progressive, some with superimposed relapses. During RRMS an underlying autoimmune pathology presides which leads to nerve damage through inflammatory processes. Therapeutic modulation of these inflammatory processes is beneficial in reducing relapses and increasing the periods between them. A key approach is to induce a switch in immune system reactivity from a proinflammatory (T helper1) to a less inflammatory (T helper2) profile. Immune system regulation is faulty in MS and reconstitution of regulatory T and B cell activity is a goal of effective intervention strategies. There is evidence that Intestinal parasite (helminth) infection is protective for MS as it induces a T helper2 profile and promotes regulatory cell activity. Deliberate, controlled helminth infection may be a useful therapeutic intervention.

In 2012, the Nottingham University Hospitals Multiple Sclerosis Clinic commenced the *Worms for immune regulation of multiple sclerosis* (WIRMS) study (NCT 01470521), which was a randomized, double-blinded, placebo controlled study of hookworm treatment of multiple sclerosis. A total of 72 patients were enrolled, 36 were infected with 25 larvae of *Necator americanus* and 36 were given placebo. At

the time of commencement, this was the largest study so far performed to assess the efficacy and safety of helminth therapy. Subsequently, concerns that helminth therapy might induce Epstein-Barr virus (EBV) reactivation have been raised and a key objective of the study presented in this thesis was to assess whether EBV reactivation is a complication of helminth therapy. Because a unique collection of serially collected samples was available together with relevant clinical information it has proven possible to investigate the time course of infection of EBV and other selected human herpesviruses (cytomegalovirus and varicella zoster virus) in helminth treated and placebo controlled patients.

Markers of EBV infection (early antigen IgG – EBV EA IgG, virus capsid antigen IgG and IgM – EBV VCA IgG and IgM, nuclear antigen-1 IgG – EBNA-1 IgG), cytomegalovirus infection (CMV IgG) and varicella-zoster virus infection (VZV IgG) were measured using commercially available enzyme-linked immunoassays (ELISAs). Samples from 51 patients (26 helminth infected and 25 placebo controlled) were available for testing and pre-treatment status was measured together with levels during the nine months of helminth infection and post infection/placebo status three months after the cessation of treatment. There was no evidence of treatment related herpesvirus reactivation in any of the patients sampled. Pre-treatment a total of 8 (30.7%) helminth treated patients were EBV EA IgG positive compared to 10 (40.0%) placebo controlled patients. The difference in the EBV EA IgG sero-positivity between the two groups was not significant and a state of

constant reactivation was maintained throughout the study period. All patients (n = 51) were EBV VCA IgG and EBNA-1 IgG positive and antibody levels were stable throughout the study. One helminth treated patient was EBV VCA IgM positive and one placebo-controlled patient was EBV VCA IgM positive and in both cases, the pre-treatment serum samples were EBV VCA IgM positive. A total of 13 (50%) helminth treated patients and 4 (16.0%) placebo control patients were positive for CMV IgG. The difference in the CMV IgG sero-positivity between the two groups was significant. Generally, the CMV IgG levels were stable throughout the study period. All patients were VZV IgG positive and VZV IgG levels were constant over the study period in all but one case.

In conclusion, there was no evidence of EBV reactivation, or reactivation of other human herpesviruses, at the therapeutic dose of *N. americanus* used. The number of *N. americanus* larvae used to infect patients may prove critical as higher doses may prove deleterious to the patient and lower doses may fail to generate an adequate immune response. Generally, antibody levels of all IgG markers were stable throughout the study period. There was a significant mismatch of CMV seropositivity between the helminth treated and placebo control groups and the reasons for this sero-discordance are difficult to explain, particularly as patient recruitment was randomized. Cytomegalovirus serostatus may influence host immune responses and so prejudice the key principle that “like” is being compared with “like” apart from the treatment given. It would be most interesting to determine if other

“randomized” clinical trials show CMV sero-discordances at the recruitment stage. Future clinical trials assessing the immunomodulatory effects of helminth infection may need to take account of patient CMV sero-status at the recruitment stage if the findings of this study are replicated. Finally, for this study the bulk of patient clinical data remains to be made available at the time of writing of this thesis, so any conclusions put forward should be viewed as preliminary.

ACKNOWLEDGEMENTS

I am grateful to my supervisor, Professor Cris Constantinescu, for allowing and supporting me to do this project. Professor Constantinescu was the originator of this study and chief investigator of the WIRMS clinical trial, which generated the samples and clinical data used herein. My thanks also to all the patients and staff involved in the WIRMS clinical trial without whom this study would not have been possible. The study/trial has been supported by the UK Multiple Sclerosis Society, an unrestricted grant from Bayer, and the Forman Hardy Charitable trust.

I would like to thank Chief Technician Mrs Nanci Frakitch for general laboratory assistance. I am also grateful to my wife Penelope for her support during my studies over the last year and for her assistance with word-processing this manuscript.

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
CD	Cluster of differentiation
CIS	Clinically Isolated syndrome
CMV	Cytomegalovirus
CNS	Central Nervous System
COV	Coefficient of variation
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
EA	Early antigen
EAE	Experimental Allergic Encephalomyelitis
EBNA	Epstein Barr Nuclear antigen
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
Foxp3	Forkhead box protein 3
GD	Gadolinium
gE	Glycoprotein E
gI	Glycoprotein I
GM-CSF	Granulocyte macrophage colony stimulating factor
HAART	Highly Active antiretroviral treatment
HCMV	Human cytomegalovirus
HHV	Human Herpesvirus

HIV	Human immunodeficiency virus
HSV	Herpes Simplex Virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
mIU/ml	Milli-International Units per millilitre
MRI	Magnetic Resonance Imaging
MS	Multiple sclerosis
OD	Optical Density
PEI	Paul Ehrlich Institute
PMS	Progressive Multiple Sclerosis
PPMS	Primary Progressive Multiple Sclerosis
PRMS	Progressive and relapsing Multiple Sclerosis
RRMS	Relapsing and remitting Multiple Sclerosis
SPMS	Secondary Progressive Multiple Sclerosis
TGF	Transforming Growth Factor
T_H	T-Helper cell
U/ml	Units per millilitre
VCAM	Vascular cell adhesion molecule
VLA	Very-late antigen
WIRMS	Worms for Immune Regulation of Multiple Sclerosis

TABLE OF CONTENTS

LISTS OF FIGURES AND TABLES

INTRODUCTION

1.1 Clinical presentation and diagnosis of multiple sclerosis

1.1.1 Historical

Descriptions of afflictions resembling multiple sclerosis can be traced back to the late 14th Century; however, it was not until the first half of the 19th century that convincing accounts of the disease and the presence of plaques in the nervous system became available (1). Reading the personal diary of Augustus d'Este (1794-1848) a disease is described commencing with attacks of visual disturbances followed by episodes of leg weakness and gait disturbance culminating with increasing spasticity (2). It was not until the second half of the 19th century that physicians could ascribe this set of signs and symptoms as multiple sclerosis. Following their appointments as "*chefs de service*" at La Salpêtrière, Paris in late 1861 (3), Jean-Martin Charcot (1825-1893) and Alfred Vulpian (1826-1887) first described multiple sclerosis in a clinico-pathological context. In a series of lectures and case presentations given between 1865-1868, their descriptions of "*la sclérose en plaques*" characterized multiple sclerosis as a distinct clinical entity enabling others to identify the same disease in their patients. Charcot's triad of intention tremor, nystagmus and scanning speech became widely adopted for diagnosis during life; however, it was only at autopsy that a more definitive diagnosis could be made.

In England, cases of "*insular sclerosis*" were first fully described during the period 1873-1875 (4). Over the next 70 years a variety of proposals were made as to the causation and distribution of the disease. For example, there were reports that multiple sclerosis was relatively common; others stated it to be relatively rare. There were reports of an association of the disease with higher latitudes; and there were reports that multiple sclerosis was more frequent in females, which were contradicted by other reports that it was more frequent in males. A review by Tracy J Putnam (5) assessed the state of medical knowledge by the early 1940s. There was little agreement amongst neuropathologists as to the aetiology of demyelinating diseases (post-infectious and disseminated encephalomyelitis, Schilder's disease, diffuse sclerosis, neuromyelitis optica and acute transverse myelitis) although all had the same underlying histopathology, differences being due to the location and intensity of lesions. An important diagnostic point was to show evidence of lesions scattered in time and space, and examination of spinal fluid greatly helped with diagnosis. Finally, the local incidence of multiple sclerosis showed an association with latitude, the disease being relatively more common in the Baltic countries, Scotland, and the North Atlantic seaboard compared to southern Europe, southern states of the USA and countries such as China.

An accurate diagnosis of multiple sclerosis is important not only in the clinical context, but is essential for the purposes of clinical trials of treatments and the understanding of the aetiology of disease. The specificity of a clinical diagnosis of multiple sclerosis was initially shown

to be highly variable. For instance, during the 1960s Charles Poser undertook a study (6) in which detailed clinical summaries of 25 autopsied cases of multiple sclerosis were sent to 190 neurologists in 52 countries. They were asked to classify the cases as probable, possible, or unlikely according to their own criteria, and 108 responses were received from 33 different countries. Following analysis, the variability in the accuracy of diagnosis ranged from 2.8% to 91.7% for cases of "pure" multiple sclerosis. By the end of the 20th Century several clinical diagnostic algorithms for diagnosis of multiple sclerosis had been proposed; however, despite the availability of laboratory techniques such as oligoclonal banding and imaging techniques such as magnetic resonance imaging (MRI) the diagnosis of multiple sclerosis still presented challenges and universally accepted criteria were lacking (7, 8).

1.1.2 Multiple sclerosis diagnosis in the 21st Century

Magnetic resonance imaging now plays a pivotal role in the diagnosis of multiple sclerosis. It is used to confirm or reject the clinical diagnosis, for monitoring of disease activity, and as a key outcome measure in clinical trials (9). In 2001, an international panel recommended diagnostic criteria (often referred to as the "McDonald criteria") for multiple sclerosis, which for the first-time integrated specific MRI evidence with clinical and other paraclinical diagnostic methods for lesion dissemination in time and space (10). Particular strengths of the International Panel MRI recommendations were that they enabled earlier diagnosis, therefore reducing uncertainty and anxiety for many

patients while at the same time the likelihood of false-positive diagnosis was reduced (11). A review of the application of the new McDonald diagnostic criteria in patients presenting with clinically isolated syndromes (CIS) suggestive of multiple sclerosis showed that within a year there was a 28% increase in confirmed diagnoses of multiple sclerosis compared with previous diagnostic criteria (12). The McDonald criteria were refined further in 2005 (13) and 2010 with progressive improvements of sensitivity to 72% without corresponding reductions in specificity, which ranged between 87% - 91% (14).

The International Panel on diagnosis of multiple sclerosis met for a third time during May 2010 in Dublin, Ireland (15). Aims of the meeting were to review the 2005 McDonald criteria in the light of new research findings, examine requirements for showing dissemination in time and space and focus on application to paediatric, Asian and Latin American populations. In England, current National Institute for Health and Care Excellence (NICE) guidelines (16) on diagnosing multiple sclerosis are based on the McDonald criteria. They stipulate that the diagnosis of multiple sclerosis should not be made on the basis of the results of magnetic resonance imaging alone and that clinical history and presentations together with risk stratification should be given due consideration.

The preceding sections show that diagnosis of multiple sclerosis and monitoring of clinical course including the identification of relapses is primarily a clinical exercise aided by the availability, in particular, of suitable imaging data. When considering the impact of treatment on

the course of multiple sclerosis it is vital that consultant neurologists highly experienced in this field of medicine undertake review of clinical effect. Despite the progress that has been made, rates of misdiagnosis of multiple sclerosis may be as high as 10% (17). There is no laboratory test available for the diagnosis of multiple sclerosis although CSF examination (total IgG and oligoclonal binding) can be deemed useful, and detection of aquaporin 4 antibodies has proved most useful for the differential diagnosis of neuromyelitis optica spectrum disorder (18). This section shows that for the purposes of understanding the aetiology of multiple sclerosis and assessing the impact of disease modifying interventions careful patient recruitment and monitoring by highly experienced physicians are required.

Multiple sclerosis is a central nervous system disease in which the myelin nerve sheath is progressively destroyed (demyelination) with associated axonal damage/loss and nerve cell death (19). The destruction is immune-mediated taking place as part of an inflammatory process (20). The disease initially presents in one of two forms; relapsing-remitting multiple sclerosis (RRMS) which is seen in approximately 85% of cases (21), or primary progressive multiple sclerosis (PPMS). In RRMS, remissions due to myelin repair, resolution of inflammation, and neural plasticity, become less frequent over time and the disease assumes a secondary progressive (SPMS) course (22).

1.2 Is multiple sclerosis an autoimmune (or infectious) disease?

1.2.1 An overview of the epidemiology and genetics of multiple sclerosis

Multiple sclerosis is the leading cause of non-traumatic neurological disability in young adults in the UK and Europe although national level data is limited, and the application of differing diagnostic criteria combined with the differing sensitivities and specificities of such criteria (see 1.1.2) hamper spatial and temporal comparisons (23). In the UK, the number of new cases over time (incidence) and the burden of disease within populations over time (prevalence) appears to be increasing (24, 25). Recently, it has been estimated that there were 126,669 (203.4/100,000) people living with multiple sclerosis in the UK and that this number represented a year on year increase of approximately 2.4% for the period 1990 – 2010 (26). Differences in the prevalence of multiple sclerosis in UK regions have been observed; for example, the rates of disease are significantly higher in Scotland (27, 28) compared to regions of England (29, 30).

From a global perspective, an estimated 2.3 million people are believed to have multiple sclerosis (31) with the highest prevalence in the USA and Europe and the lowest prevalence in Africa, Asia, and South America. For many of the purportedly low-prevalence regions the quality of the epidemiological data is limited (32) and prevalence rates may be significantly higher than initially predicted (33, 34). Multiple sclerosis is generally recorded two to three times more frequently in

females (35) and increases of prevalence in this population have been observed to be a driver for the increased overall prevalence of multiple sclerosis (36, 37). Several studies (35, 38) have noted that the marked female sex-linkage is apparent for relapsing-remitting multiple sclerosis and that it appears to be absent or much reduced in primary progressive disease.

There is increasing evidence that genetic, environmental, and socio-economic factors have a role in the development of multiple sclerosis (39, 40). Possible explanations for the spatial distributions of multiple sclerosis include intrinsic population genetic susceptibilities (41), levels of vitamin D (42), lack of exposure to UV radiation (43), and adoption of "Westernized" lifestyles (44). Further discussion of these factors is beyond the scope of this thesis; however, infections represent another major risk factor and will now be discussed.

1.2.2 Does multiple sclerosis have an infectious cause?

Multiple sclerosis is generally recognized as an autoimmune mediated disease in which demyelination and axonal death is immune mediated (45); however, there is increasing evidence of ongoing neurodegenerative processes even in the early stages (46). Autoimmune diseases typically show an interaction of genetic and environmental factors to varying extents (47). However, an alternative view has been proposed (48, 49) suggesting that multiple sclerosis is an infectious process. Several lines of evidence have been quoted in support of this view including the discordance of multiple sclerosis in identical twins, altered humoral and cell mediated responses, migration

studies and recurrent outbreaks of disease. Familial aggregation studies (50) have shown an increased recurrence risk of multiple sclerosis in monozygotic twins (25-30%), and a reduced recurrence risk is apparent in half siblings (1.32%) compared to full siblings (3.46%) against an estimated general population and adopted sibling recurrence risk of 0.2%. Most monozygotic twins are discordant and although many allelic associations have been identified in genome analysis studies (51) there still remains a lack of a definitive genetic marker for multiple sclerosis. Oligoclonal banding is seen in many cases of multiple sclerosis with the conclusion that intrathecally-produced immunoglobulins contribute to the disease process (52) and it has been suggested that the IgG manifest in such bands is produced in response to the causative agent of multiple sclerosis in a similar manner to specific IgG produced intrathecally during infectious encephalitides (53). In general (54), migrant studies, despite their inherent limitations, have shown that population groups living in areas of higher multiple sclerosis risk see a reduction in risk of multiple sclerosis following migration to areas of lesser multiple sclerosis risk, and there is limited evidence that moves from low risk areas to higher risk areas result in an increased risk of multiple sclerosis. Furthermore, it appears that this phenomenon is most prominent in individuals who migrate during the first two decades of life. Finally, compelling epidemiological evidence, which frequently has been given in support of an infectious cause of multiple sclerosis, is a series of outbreaks of multiple sclerosis that

occurred in the Faroe Islands following an occupation by British troops during the 1940s (48, 55).

Several microbial agents have been proposed, particularly those responsible for childhood infection, as having the potential for playing a significant role in the development of multiple sclerosis; however, definitive evidence remains to be provided (56). Potentially, significant microbial associations have been reported for Epstein Barr virus (57), *Chlamydia pneumoniae* (58), and human endogenous retroviruses (59). The role of human herpes viruses in multiple sclerosis is reviewed in section 1.4.

1.2.3 The Hygiene Hypothesis

Autoimmune diseases are a group of diseases characterized by a loss of immunological tolerance to self-antigens. In most cases, the development of autoimmune disease is a product of a complex interaction between intrinsic susceptibility (or protection) mediated at the genetic level and external or environmental factors (60, 61). The spatial distribution of autoimmune diseases has been shown to be highly variable (62) and temporal trends are disease specific; for example, childhood type 1 diabetes has increased worldwide during the 20th Century (63), although this trend is not apparent for rheumatoid arthritis (64). The Hygiene hypothesis was initially proposed to explain the increasing incidence of allergic diseases and was subsequently shown to be equally applicable in accounting for the increasing incidence of autoimmune diseases (65).

In the first instance, the Hygiene Hypothesis made the proposition that reduced exposure to infections during childhood led to an increase in allergic diseases (66). Subsequently, spatial variations in allergic and autoimmune diseases were shown to be related to socioeconomic conditions (67) so that their frequency was increased in populations with high economic status (eg. Northern Europe) and decreased in populations with lower socioeconomic status (eg. Southern Europe). Animal studies, epidemiological studies, and migration studies have both proven useful in providing additional evidence in support of the Hygiene Hypothesis. Studies (68) of non-obese diabetic mice have shown that housing conditions and exposure to virus infections are key determinants in the development of type 1 diabetes. Finland, an affluent country, has the highest incidence of type 1 diabetes in the World whereas in the neighbouring, less affluent, Russian Karelia the incidence of type 1 diabetes is significantly lower, and this is believed to be linked to the differing microbial challenges prevalent in each country (69). Finally, a review (70) of multiple sclerosis cases in Gothenburg, Sweden showed that an Iranian subpopulation, which migrated mainly during the 1980s and 1990s, manifested an increased risk of multiple sclerosis compared of their birthplace, approximating to that of the indigenous Swedish population.

1.2.4 The impact of parasite infection on multiple sclerosis and the Hygiene Hypothesis

A tenuous correlation has been observed (71) between the decline of certain bacterial (eg. rheumatic fever and tuberculosis) and

virus (eg. measles, hepatitis A) infections and the rising incidence of certain autoimmune diseases (eg. multiple sclerosis, type 1 diabetes). Low levels of allergic and autoimmune diseases have been associated with countries experiencing high levels of helminthic infestations and common factors are inadequate sanitation, poor diet, and low socioeconomic status (72, 73). Following anti-helminthic eradication programmes increased population incidences of allergic and autoimmune diseases have been reported (74). Additionally, allergic reactions have been shown to recur in individuals following successful anti-helminthic eradication treatment (75). Immunologically, helminth infestation generates a complex immune response involving both adaptive and innate pathways. A broadly anti-inflammatory T_H2 response is generated characterized by elevated levels of associated cytokines (eg. IL-4, IL-5, IL-10, IL-13, TGF-β), suppression of T_H1 inflammatory cytokines (eg. IFN-γ) and modified regulatory B and T cell activity (76, 77).

In the context of multiple sclerosis, there is considerable evidence (78) implicating the increasing incidence of multiple sclerosis to be inversely correlated with a decreasing prevalence of helminth infestation. Epidemiological studies (79) and studies of helminth infection in animal models of multiple sclerosis (80) have generated evidence supporting the suggestion of an interaction between helminth infestation and multiple sclerosis. Furthermore, human studies have shown that helminth infection can immunomodulate the relapsing and remitting form of disease (81) through increased production of IL-10

and TGF-β with induction of $CD25^{+}CD4^{+}FoxP3^{+}$T cells and that this effect is reversed following helminth eradication (82).

1.3 Treatment strategies for multiple sclerosis

1.3.1 The early days of treatment

> *"It would be nice if a physician from London, one of these days, were to gallop up hotspur, tether his horse to the gait post and dash in waving a reprieve –the discovery of a cure!"*

Excerpt from "The Journal of a disappointed Man" by B. F. Cummings (1889-1919) who wrote under the literary name of W. N. P. Barbellion. Source T. Jock Murray, Multiple Sclerosis (83).

The late 19th Century and first half of the 20th Century heralded major advances in the diagnosis and treatment of infectious diseases. For example, during the first half of the 1914 – 1918 War tetanus was a major cause of mortality in wounded troops; however, the swift implementation of mass tetanus vaccination of troops and adequate wound toilet resulted in the virtual elimination of tetanus from 1916 onwards (84). Paul Ehrlich was the first to develop non-poisonous, effective antibacterial agents (eg. Salvarsan) and during the late 1920s Sir Alexander Fleming was in the process of discovering penicillin. The capacity to identify the aetiological agent of disease enabled the process of learning how to diagnose and treat that disease. It is, therefore, not surprising that scientific opinion was biased towards treating multiple sclerosis as an infectious disease in the hope that a cure might be found. An address (85) given by Douglas McAlpine on disseminated sclerosis in 1927 encapsulated the prevailing views of the time as exemplified by the following excerpts from the published paper.

"Further clinical evidence is afforded by the characteristic relapses and remissions which occur during the course of the disease in the majority of cases. Any theory which attributes the condition to developmental errors falls to the ground in the presence of the curious variations in the clinical picture. In this respect disseminated sclerosis resembles meningo-vascular syphilis, pulmonary tuberculosis, and other chronic infections. The variations in the intensity of the infective process can be best explained by supposing that a virus, or its toxin, periodically invades the central nervous system, so producing a train of symptoms which will depend on the particular part of the neuro-axis attacked."

Excerpt from an address given by Douglas McAlpine, M.D., M.R.C.P., to the Oxford Division of the British Medical Association, published in the Lancet, February 12, 1927 (85).

While the prevailing view that the aetiology of multiple sclerosis was infectious the choice of treatments adopted reflected experience with genuine infectious diseases and, consequently, yielded little benefit for the patient. In 1950, a series of articles published in the Journal of the American Medical Association (86, 87) by George A. Schumacher reviewed the state of past treatment options as follows.

"Numerous modes of therapy have become obsolete despite original contentions of their value. Among these use of antiseptics, especially arsenic, fever therapy, various vaccines,

and serums, autohemotherapy, belladonna, endocrine substances and penicillin. It may be safely stated that these measures have proved largely ineffective."

Excerpt from a paper (86) by G. A, Schumacher, JAMA 1950; 143: 1146-1154.

During the 1950s there was a move away from infection as a cause of multiple sclerosis towards "allergic theory" and the consideration of multifactorial causation (88). Henry Miller and Kurt Schapira in an article (89) published in the British Medical Journal described allergic theory thus.

"As we see it, what may be broadly termed the allergic hypothesis may be briefly developed in the following terms. The initial lesion in multiple sclerosis appears to be focal, disseminated microscopic perivenous exudation and oedema. This is accompanied or followed by an inflammatory reaction of variable intensity at the same site, which is in turn succeeded there by the well-defined demyelination and glial proliferation typical of the established disease...there is good reason to believe that the initial perivenous lesion which is the first visible pathological sign of the disease represents in itself the larval manifestation of neural hypersensitivity"

Excerpt from a paper (89) by Henry Miller, M.D, F.R.C.P. and Kurt Schapira, M.B. published in the British Medical Journal, March 28, 1959.

The recently established experimental allergic encephalomyelitis model in animals had shown that injection of the sterile extracts of animal nervous tissue could result in the development of analogous lesions (90); therefore, the direct involvement of infectious agents was equivocal. Around this time steroids had been shown to have a positive benefit in the treatment of rheumatoid arthritis (91) and the first trials of steroid treatment of multiple sclerosis were undertaken; however, the results were disappointing (92). Despite the initial setbacks, steroid treatment has now become an established treatment for relapse; however, although recovery can be hastened by their use, they have little impact on the final degree of recovery or the overall progression of disease, and optimum treatment regimens still remain to be determined (93, 94).

1.3.2 Disease modifying treatments for multiple sclerosis

Disease modifying treatments have been shown to influence the course of relapsing and remitting multiple sclerosis; however, they have little or no effect on the progressive form of the disease (95). An extensive review of disease modifying treatments, established or under development, is beyond the scope of this thesis and only some specific comments will be made relating to established first-line treatments, newly available oral drugs, and monoclonal antibody based therapeutics.

The first widely used and effective and relatively non-toxic disease modifying treatment to become available was interferon β.

Initially, the interferons had been discovered as agents that inhibited influenza virus multiplication (96) and had been of interest because of their antiviral activity; however, during the 1980s their role in the potential treatment of multiple sclerosis was assessed. Interferon γ treatment was associated with an increased rate of exacerbations; however, trials with interferons α and β yielded promising results (97). In these early studies, purified interferon preparations were used which were difficult and costly to produce. Genetic engineering resolved this difficulty; for instance, interferon β-1b has been produced which retains the activity of the natural form but has greater stability due to the replacement of a cysteine residue by a serine residue at position 17, and it is not glycosylated. Alternative forms of interferon β (IM IFN-β1a & SC IFN-β1a) comprising the native amino acid sequence are also available giving a choice of route of administration (98). The costs of standard treatment packages of interferon β-1b (250 μg SC), IM IFN-β1a (30 μg) and SC IFN-β1a (44 μg) are approximately €800 monthly (99). Development of flu-like symptoms and local skin reactions are common adverse events/side effects together with depression, and the production of neutralizing antibodies which may be problematic; however, numerous clinical trials have established efficacy and a reasonable long-term safety profile (100, 101).

A survey (102) of current European treatment practices for the initial stages of relapsing and remitting multiple sclerosis showed 37% of neurologists to use interferon β preparations, 25% to use dimethyl fumarate, 22% to use teriflunomide, and 9% to use glatiramer acetate,

as first line therapy. Dimethyl fumarate is a fumaric acid derivative (103) that is metabolized in the gastrointestinal tract to monomethyl fumarate – the active component. Initially, it was used for the treatment of psoriasis; however, *in vivo* studies (104) in the experimental allergic encephalomyelitis (EAE) model showed potential for the treatment of multiple sclerosis. Subsequent clinical trials have shown administration of the drug to significantly reduce relapse rates through yet incompletely elucidated mechanisms of action that include induction of a shift to aT_H2 immune response (105). Obviously, an attractive therapeutic agent because it can be taken orally; however, a recent post-approval study has raised concerns of a comparative lack of efficacy and discontinuation due to lymphopenia and gastrointestinal side effects (106). Finally, there have been reports of progressive multifocal leukoencephalopathy in patients treated with this drug (107). Teriflunomide, like dimethyl fumarate can be taken orally. It is an anti-inflammatory agent, which was initially used to treat another autoimmune disease (rheumatoid arthritis) that has subsequently been shown in EAE animal models to have potential efficacy for the treatment of multiple sclerosis (108). It is an active metabolite of leflunomide that inhibits dihydroorotate dehydrogenase activity, which is essential for the pyrimidine synthesis required by rapidly proliferating, activated lymphocytes (109). Teriflunomide has also been shown to display pyrimidine synthesis independent effects on the immune system including the disruption of T cell interactions with antigen presenting cells and modulation of cytokine activity towards the induction of a T_H2

response (110). Two major placebo-controlled clinical trials have been conducted to determine the efficacy of teriflunomide in patients with relapsing multiple sclerosis (111) and these showed significant dose-dependent reductions in annualized relapse rates ($p<0.001$) and disability scores ($p<0.1$). The drug is teratogenic and side effects include hair loss and raised liver transaminases (112).

We are now in an era where targeted biotherapeutics can be designed and produced for specific immunological pathways. The production of monoclonal antibodies using hybridoma technology, first described by Köhler and Milstein in 1975 (113), enabled limitless quantities of reproducible and specific immunoglobulins to be produced from immortalized cell lines. Unfortunately, the therapeutic use of such molecules was greatly limited because the human immune system recognized them as “foreign”. Advances in antibody engineering have made possible the production of therapeutic monoclonal antibodies by “humanizing” adaptations combined with the application of other bioengineering structural modifications to improve pharmacokinetics (114). Consequently, a range of therapeutic monoclonals have been produced for a variety of applications including the treatment of multiple sclerosis (Table 1) raising the possibility of major advances in the treatment options available for this disease (115).

Table 1. Therapeutic monoclonal antibodies and their targets for relapsing and remitting multiple sclerosis treatment.

Therapeutic monoclonal	Principal mechanism of action	Concerns and limitations
Alemtuzumab – humanized anti-CD52 (116)	Immunomodulation through the depletion of B and T cells and subsequent changes to subsets upon repopulation.	Risk of herpes virus infections following administration. Increased risk of development of autoimmune conditions.
Daclizumab – humanized anti-CD25 (117)	Immunomodulation through a number of effects following binding to the interleukin-2 receptor including the expansion of regulatory natural killer cells.	Increased risk of infections, elevated liver enzymes and skin reactions. Possible induction of autoimmune conditions.
Natalizumab – humanized anti-α4β1 integrin antibody (118)	Disrupts leukocyte migration to sites of inflammation by blocking the VLA4 integrin receptor present in leukocytes thereby preventing binding to activated adhesion (VCAM-1) receptors expressed by vascular endothelium cells.	Major safety concern is the risk of progressive multifocal leukoencephalopathy.
Ocrelizumab – humanized anti-CD20 (119) Rituximab used as a treatment for rheumatoid arthritis is another anti-CD20 antibody with application against multiple sclerosis (120)	Depletes B cells except early precursor and plasma forms (which do not express CD20) through antibody-dependent cellular cytotoxicity and complement-mediated lysis.	Immunosuppressive and decreased immunogenicity (vaccination contraindicated). Increased risk of infections, particularly respiratory tract. Herpes viruses reactivations. Infusion related reactions. Concern over potential increased risk of malignancy.

1.3.3 Helminth therapy – an alternative option for treating multiple sclerosis

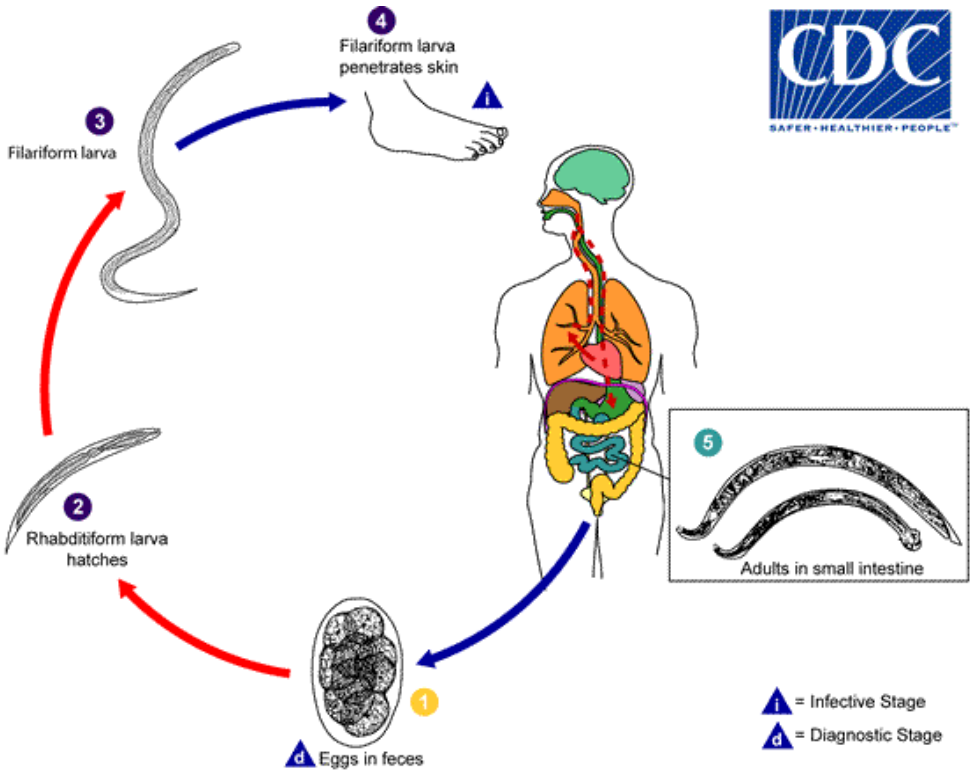
Helminth therapy was first reported to be effective in the treatment of human inflammatory conditions in 2005 (121). Subsequent clinical trials yielded variable results in relation to efficacy although tolerability was generally good (122). Several EAE mouse model studies (123, 124) have shown that helminths, or their products, have potential as treatments for multiple sclerosis. Epidemiological and observational data (see section 1.2.4) also support the potential application of helminth therapy in the treatment of multiple sclerosis. There are limited clinical trial data (<100 patients) that generally support further clinical investigations of helminth therapy for multiple sclerosis and these have been reviewed by Tanasescu and Constantinescu (125).

The University of Nottingham and Nottingham University Hospitals have undertaken the largest clinical trial (NCTC1470521) of helminth therapy for multiple sclerosis, to date. In this study (WIRMS), which is randomized, placebo-controlled, and double-blind in design, 36 patients presenting with a range of forms of multiple sclerosis (predominantly relapsing and remitting) have been infected with *Necator americanus* (human hookworm) and a similar group have received placebo. The trial period was for one year during which time patients were closely monitored and at nine months they received mebendazole to eradicate helminth infection. The primary measure of efficacy was based on the cumulative number of new or enlarging

Gadolinium-enhanced lesions after nine months. Samples were taken at defined periods of the trial for the measurement of biochemical, immunological, and parasitological parameters.

There are significant concerns with the application of helminth therapy relating to safety, efficacy, and other factors (126). Parasites such as *N. americanus* are human pathogens themselves. The life cycle of *N. americanus* is shown in figure 1 and immediate safety concerns relate to the risk of pulmonary damage during larval transit, anaemia following gastrointestinal blood loss and adverse effects on airways responsiveness (127). Efficacy will be dependent on various factors (128); for example, commencement of treatment in the early stages of multiple sclerosis appears to yield the best results. Additionally, establishment of the longer-term (> 1 year) benefits or limitations of treatment is needed. Optimisation of dosing schedules and the helminth load to be administered is required. The reproducibility and safety of manufacturing processes remains to be established. Finally, the use of helminth products instead of live helminth treatment requires evaluation at many levels ranging from identifying the immunological mechanisms involved (124, 129, 130) to final optimal clinical usage.

Figure 1. Life cycle of *Necator americanus*



Legend.

Eggs (1) are passed in the stool and larvae hatch under favourable conditions. The rhabdatiform larval form (2) matures in the soil or faeces. The infective filarial form (3), on contact with the human host (4) penetrates the skin and is transported via the blood to the heart and then to the lungs. Following transpulmonary migration larvae ascend the bronchial tree to the pharynx and are swallowed. The larvae attach to the wall of the small intestine and mature into adults (5). This stage is associated with intestinal blood loss.

Life cycle image courtesy of CDC Division of Parasitic Diseases and Malaria and is supplied free of copyright as an educational resource (see www.cdc.gov/parasites/.)

1.4 Human herpesviruses and multiple sclerosis

1.4.1 The Human herpesviruses

Over 100 species of herpes viruses have been identified and they have been isolated from a wide variety of hosts including humans, non-human primates, other mammals, birds, amphibians and reptiles (131). Common features of these viruses include a linear, double-stranded DNA genome, contained within an icosahedral capsid structure, together with the possession of a tegument and lipid envelope, and the capacity to establish host latency. In recent years (132), the taxonomy of the herpes viruses has been updated with the establishment of the order *Herpesvirales* comprising three families – *Herpesviridae* (avian, mammalian, and reptilian viruses), *Alloherpesviridae* (amphibian and piscine viruses) and *Malacoherpesviridae* (molluscan viruses). Within the family *Herpesviridae* there are three subfamilies – *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* and human herpes viruses are represented in each of these subfamilies (133). There are eight human herpesviruses (Table 2) and in this study the serological profiles from a representative of each subfamily (HHV-3, HHV-4 and HHV-5) have been determined. The financial and time constraints of the project influenced this approach together with the fact that previous studies have shown the selected viruses to be of significant interest. Pros and cons for investigating the serological profiles of the other human herpesviruses (HHV-1, HHV-2, HHV-6, HHV-7 and HHV-8) are discussed in section 5.4 (pt 5) which addresses potential future work.

Table 2. The human herpesviruses

Taxon name/ **Subfamily**	Genus	Common name/ Clinical condition(s)
Human herpesvirus 1 ***Alphaherpesvirinae***	*Simplexivirus*	Herpes simplex virus type 1 (HSV-1). Primary infection: Herpetic skin infections (eg. stomatitis), conjunctivitis, encephalitis. Reactivation: cold sores. HSV-2 may cause these also.
Human herpesvirus 2 ***Alphaherpesvirinae***	*Simplexivirus*	Herpes simplex virus type 2 (HSV-2). Primary and recurrent genital herpes. Neonatal herpes. HSV meningitis. HSV-1 may cause these also.
Human herpesvirus 3 ***Alphaherpesvirinae***	*Varicellovirus*	Varicella-zoster virus (VZV). Primary infection: Chickenpox (varicella). Reactivation: Shingles (zoster).
Human herpesvirus 4 ***Gammaherpesvirinae***	*Lymphocryptovirus*	Epstein-Barr virus (EBV). Infectious mononucleosis (glandular fever). Lymphoproliferative diseases (eg. post-transplant) Lymphomas (eg. Hodgkin).
Human herpesvirus 5 ***Betaherpesvirinae***	*Cytomegalovirus*	Human cytomegalovirus (CMV). Congenital infection. Mononucleosis syndrome. Post-transplant infections (primary or recurrent) eg. pneumonitis.
Human herpesvirus 6 ***Betaherpesvirinae***	*Roseolovirus*	Human herpes virus 6A (HHV-6A) and 6B (HHV-6B). Primary infection: fever and/or roseola /sixth disease. In some cases, encephalitis. Reactivation: post-transplant Illness including encephalitis.
Human herpesvirus 7 ***Betaherpesvirinae***	*Roseolovirus*	Human herpes virus 7. Primary infection: fever and/or roseola. Neurological sequlae including seizures.
Human herpesvirus 8 ***Gammaherpesvirinae***	*Rhadinovirus*	Human herpes virus 8. Kaposi sarcoma.

1.4.2 *Human herpesvirus 3* (Varicella-Zoster virus)

Commonly known as Varicella-Zoster virus (VZV), human herpesvirus 3 is an alpha herpesvirus. The virus genome of approximately 125,000 base pairs (125 kbps) encodes at least 70 genes and comprises a unique long region (100 kbp) and unique short region (5.4 kbp) flanked by internal and terminating repeat regions (134). The virus shows very low genetic diversity on comparison with other herpes viruses (135). Based on internationally agreed criteria and nomenclature (136) five VZV clades (1, 2, 3, 4, and 5) have been designated together with several provisional clades (VI, VII, VIII and IX); however, in clinical, serological and virological properties they can all be considered the same. Structurally, the VZV virion is of variable size (120nm – 300nm) due to the varying thickness of the tegument and the virus envelope comprises host cell membrane with virus derived glycoprotein spikes projecting on its surface, (137). Several surface glycoproteins have been described that display a range of functions key to virus pathogenicity; for instance, glycoproteins gE and gI interact to present the virus Fc receptor and glycoproteins B and C have a role in cell attachment and virus absorption (138).

Primary infection with VZV manifests as chickenpox, and by age 10 years most English and Welsh children have been infected (139). Chickenpox is typically a self-limiting infection of childhood in immunocompetent children; however, in adults, pregnant women, and the immunodeficient, complications of chickenpox (eg. varicella

pneumonia, encephalitis) can result in significant morbidity and even mortality (140). Transmission of VZV is principally by the respiratory route through aerosols (141) and the vesicles produced during the infection initially contain high titres of cell-free virus and represent an infection risk. Initially, VZV infects the respiratory epithelia of the upper respiratory tract; in particular, tonsillar lymphoid tissue where T cells are preferentially infected (142) which then enter the circulation generating a short-lived viraemia. Dissemination of the virus leads to infection of cutaneous epithelial cells, where local immune responses are, initially, insufficient to prevent extensive virus multiplication leading to vesicle formation. At some stage, neurons of the sensory ganglia are infected and virus latency established. Zoster, or shingles, results when latent virus reactivates; hence, the characteristic dermatomal distribution of the rash. The precise factors responsible for virus reactivation remain to be fully described; however, zoster is associated with a general decline in immune function and is characteristically seen in the later years of life in immunocompetent individuals (143). Alternatively, zoster can be a complication of immunodeficiency or processes leading to immune suppression in individuals of any age (144).

Several studies have reported an association of a history of VZV infection with multiple sclerosis. Ross and colleagues (145) have observed that the geographical and temporal prevalences of multiple sclerosis and varicella appear to be linked and in a control study, Rodríguez-Violante and co-workers (146) have reported an increased risk of association of a history of varicella and having multiple sclerosis.

Other studies (147) have failed to yield sufficient evidence in support of a significant relationship between VZV infection and multiple sclerosis. While an association between varicella or zoster and the development of multiple sclerosis is a matter for debate there is ample evidence that certain treatments for multiple sclerosis predispose to VZV infection and reactivation. For instance, treatment with fingolimod has resulted in fatal disseminated primary VZV infection (148) and treatment with alemtuzumab has been shown to give a higher rate of herpes zoster compared to interferon beta-1a (149).

1.4.3 *Human herpesvirus 4* (Epstein-Barr virus)

First described in 1964 following electron microscopy of cultivated human lymphoblasts from Burkitt's malignant lymphoma (150), Epstein-Barr virus (EBV) is a gamma herpesvirus (151), designated *Human herpesvirus 4*. The EBV virus genome of double-stranded DNA is linear in the virus particle, but is circularized as an episome within the nuclear compartment of infected cells. The complete nucleotide sequence of EBV is approximately 172,000 base pairs and two virus subtypes have been described, EBV1 and EBV2 that show close co-linear sequence alignments with each other (152, 153) apart from a number of known diverged alleles (154). In terms of organization, there are terminal, tandem-repeat regions (joined during circularization) separated by unique short and unique long regions interspersed with four internal repeat regions (155).

The expression of virus proteins varies between the latent and lytic phases of virus growth (156). Primary EBV infection commences with infection of oropharyngeal epithelial cells via saliva containing shed virus through complex pathways (157). EBV infection of B cells is much more efficient and virus binds to specific receptors on the cell surface; for example, the virus glycoprotein gp 350 (158) binds to a B-lymphocyte membrane receptor (CD21) which also binds complement C3d (159). Following entry into epithelial or B cells by virus endocytosis, a number of replicative strategies and interactions with the host immune system can then follow resulting in lytic infection or the establishment of latency (160). In EBV-transformed lymphoblastoid cell lines, a number of latency states (types 0, I, II, or III) have been described and these reflect the differing levels of expression of virus latency-associated proteins by infected B cells (161). These proteins include the Epstein-Barr nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA- 3B, EBNA-3C, and EBNA-LP) and latent membrane proteins (LMP1, LMP2A, and LMP2B). EBNA-1 is a DNA binding nuclear phosphoprotein and it is expressed in all latency types, and has multiple and important roles in EBV infection. Key functions of EBNA-1 include roles in the replication and mitotic separation of EBV episomes, the activation of other latency associated genes, and in lytic infection it acts as a gene expression promoter (162). Another important aspect of EBNA-1 expression is that EBNA-1 specific T cells have been shown to recognize myelin basic protein (163) raising the possibility of molecular mimicry (164). EBNA-1 is expressed in latency states I, II and III

whereas EBNA-2 is expressed in latency states II and III and the EBNA-3 proteins are expressed in latency state III. Young and Rickinson (165) have reviewed the roles and functions of these and other relevant proteins, which will not be described here. Ultimately, irrespective of the latency state adopted, EBV maintains persistence throughout life in memory B cells (sIg^+, IgD^{neg}) only (166). During initial infection (or intermittent shedding) EBV can be shed by both B cells and epithelial cells (167); however, episodes of reactivation follow the induction of the lytic phase in B cells and are accompanied by the expression of approximately 80 lytic phase specific genes. This highly complex process, involving early and late lytic proteins of varying immunodominance and in which the CD8 T cell response plays a key role, has been comprehensively reviewed by Hislop and colleagues (168).

Epstein-Barr virus infection usually occurs during childhood and in most cases, is asymptomatic (169). Marked differences in the age of acquisition, related to race and socioeconomic factors, have been reported (170) and there is an increasing tendency towards higher ages of acquisition in England and Wales (171). Infection during adolescence and adulthood is more likely to present as infectious mononucleosis (glandular fever), which typically presents as pharyngitis and cervical lymphadenopathy, frequently with hepatitis, and with reported severe sore throat, fatigue, fever and headache (172). In a small number of cases primary infection does not resolve and chronic active disease evolves which carries a high risk of significant morbidity

and mortality (173). EBV is an oncogenic virus and is primarily associated with B-cell lymphomas and nasopharyngeal carcinoma (174), which are uncontrolled proliferations of B-lymphocytes and tonsillar epithelial cells, respectively, which are the cells it initially infects. Critical to control of EBV reactivation is the suppression of EBV reactivation by the host immune system (175). Compromise of host immune function either through genetic defect, disease (eg. AIDS, malaria), or immunosuppressive treatment, such as that used for transplantation, carries an increased risk of neoplasm generation. For instance, in X-linked lymphoproliferative disease (XLP) triggered by EBV infection, 70% mortality has been reported by age 10 years, which is due to mutations of the *SH2D1A* gene resulting in fulminant infectious mononucleosis, dysgammaglobulinaemia and lymphoma (176). While XLP is a rare primary immunodeficiency, post transplant lymphoproliferative disorder (PTLD) is a significant risk of the induced immunosuppression required to prevent rejection of transplanted organs or tissues (177). The degree of risk of PTLD has been shown to be highly associated with the extent and severity of immunosuppression induced (178). EBV is also associated with tumour formation in immunocompetent individuals through, as yet, incompletely explained mechanisms. In nasopharyngeal carcinoma there is a complete association with EBV and in Hodgkin's lymphoma there is an approximately 40% association (179).

The association of EBV infection with several autoimmune diseases (eg. systemic lupus erythematosus, rheumatoid arthritis,

primary Sjögren's syndrome) has been reported (180). In the case of multiple sclerosis in adults, virtually 100% linkage has been reported with the detection of serological markers, particularly EBNA-1 IgG, of past EBV infection (181). It has also been shown (182) that the risk of multiple sclerosis developing in EBV seronegative individuals is very low. A problem with assessing the relevance of serological markers of past infection with EBV in adults is that the sero-prevalence in control subjects is also usually very high, approximately 95% by age 30 years (183). In children, the control population EBV seroprevalence is lower and a comparable risk for the development of multiple sclerosis to adults has been found (184). A history of infectious mononucleosis is positively associated (relative risk 2.17; 95% CI: 1.97-2.39) with the development of multiple sclerosis (185). High levels of EBNA-1 IgG have been shown to be associated with an increased risk of development of multiple sclerosis (186) and several studies (187-189) have shown that levels of EBNA-1 IgG are elevated in individuals with multiple sclerosis compared to controls. There have been reports that serum levels of EBNA-1 IgG correlate with multiple sclerosis disease activity. In a study (190) by Farrell and colleagues, 50 clinically isolated syndrome (CIS), 25 relapsing-remitting multiple sclerosis (RRMS), and 25 primary progressive multiple sclerosis (PPMS) patients were monitored for EBNA-1 IgG plus other EBV infection markers over a period of five years. Multiple sclerosis disease activity was assessed by the serial monitoring of gadolinium-enhanced (Gd+) lesions. EBNA-1 IgG levels were significantly elevated ($p<0.001$) in the RRMS group

compared to the CIS and PPMS groups and there was a degree of positive correlation (r=0.3) between EBNA-1 IgG and EBNA-1 IgG levels. In another study (191), 87 multiple sclerosis patients were followed for two years and multiple sclerosis disease activity was monitored by Gd+ scans. Higher EBNA-1 IgG levels were associated with increased disease activity over the total study period ($p<0.05$). Other studies have reported no association between EBNA-1 IgG levels and multiple sclerosis disease activity at least in the early stages of disease (192, 193).

EBV reactivation can have serious complications in the immunocompromised host, as described earlier. In multiple sclerosis, several studies have reported EBV reactivation to be associated with disease activity. For instance, Wandinger and colleagues (194) followed 19 multiple sclerosis patients monthly for one year and detected virus markers of reactivation in 72.7% of patients with exacerbations and in none with clinically stable disease. Both Buljevac and colleagues (195) and Latham and colleagues (196) have provided limited evidence that EBV reactivation is associated with episodes of relapse. A number of other studies (193, 197-199) have presented evidence that there is no association of EBV reactivation with multiple sclerosis disease activity. Clearly, the impact of EBV reactivation on multiple sclerosis disease activity requires further investigation.

1.4.4 *Human herpesvirus 5* (Human Cytomegalovirus)

Human cytomegalovirus (HCMV) is a betaherpesvirus and this group of herpesviruses typically shows tropism for the salivary glands, grows slowly in cell culture, and exhibits strict species specificity (200). Structurally, HCMV virions are 120-200 nm in diameter with an enveloped, icosahedral capsid containing the linear double-stranded DNA genome. The genome is the largest of the human herpesviruses comprising approximately 235,000 base pairs and housing a complement of 165 genes (201). At least 80% nucleic acid homology exists among HCMV strains (202); however, HCMV isolates display considerable antigenic and genotypic diversity (203). HCMV infects a wide variety of cell types and displays both endothelial and leukocyte cell tropisms (204). Virus latency is established in cells of myeloid lineage and virus reactivation has been shown to follow allogeneic T-cell stimulation of latently infected monocytes (205). In endothelial and epithelial cells low-level virus shedding has been shown to last for years and mechanisms of latency, reactivation or chronic infection remain to be defined or excluded (206).

In immunocompetent children and adults, HCMV infection typically is not accompanied by clinically obvious disease. HCMV is found worldwide and seroprevalence increases with age (207). Higher seroprevalences have been reported in developing countries/regions of lower socioeconomic status (208). In England and Wales, HCMV seroprevalence has been reported to range from approximately 15% in those aged 1-4 years, 30-50% in those aged 30-40 years, and approximately 80% in those aged 65 years, or older (209). HCMV is a

major cause of congenital infection and 10-15% cases are symptomatic, commonly presenting with clinical signs including hepatosplenomgaly, and neurological development abnormalities (210). Sensorineural hearing loss is a major complication of HCMV congenital infection occurring in 30-40% of symptomatic cases and 5-10% of asymptomatic cases (211). HCMV mediated disease either following primary infection, or reactivation, or reinfection is a major cause of morbidity and mortality in immunocompromised individuals. Before the effective control of HIV infection by highly active anti-retroviral therapy (HAART), HCMV retinitis, gastroenteritis or neurological disease were frequent and severe complications in individuals with low (<100/µl) CD4 cell counts (212). These days, severe HCMV infection is most frequently encountered in organ or stem cell transplant recipients at the height of their immunosuppression usually >90 days post transplant (213).

Unlike EBV infection, based on seroprevalence data there appears to be no linkage between the development of multiple sclerosis and HCMV infection (214, 215). In fact, it has been suggested that HCMV seropositivity is negatively associated with the development of multiple sclerosis in children (216) and adults (217). Recently, it has been reported that CMV infection is associated with neurodegeneration (218) and exacerbates autoimmune mediated neuroinflammation (219). It has been suggested (220, 221) that HCMV infection drives the expansion of $CD4^{+}CD28^{null}$ cells, which are highly pro-inflammatory and less susceptible to suppression by T regulatory cells resulting in the

potential for autoreactive attack and destruction of myelin. Milovanovic and colleagues (222), who investigated murine cytomegalovirus infection, have presented additional evidence supporting the contribution of CMV infection to the exacerbation of autoreactive processes. Several studies have reported an immunomodulatory effect related to CMV infection and the only common denominator is that this is a controversial topic and that further studies are required to help elucidate the impact of HCMV infection on multiple sclerosis (223).

2.0 RATIONALE, AIMS, OBJECTIVES, AND STUDY PLAN

2.1 Study rationale

Worldwide it is estimated that there are 2.3 million cases of multiple sclerosis (31) and it is one of the leading causes of disability in adults. The disease arises in genetically predisposed individuals as a consequence of environmental exposures (see section 1.2.1); however, although associations have been identified (eg. Epstein-Barr virus infection, vitamin D levels, socio-economic status, smoking) its causation remains to be identified. New disease-modifying treatments are increasingly available (see section 1.3.2) and although they are effective in reducing the number and rate of relapses they are not curative. Unfortunately, the treatments available are expensive and have potentially serious side effects (224). Helminth therapy represents an alternative approach in that it is inexpensive and potentially preventative (see section 1.3.3). As with any new therapeutic approach efficacy has to be established and potential side effects identified.

In 2012 the WIRMS study commenced at Nottingham University and hospitals and this was a randomized, double-blinded, placebo controlled study of hookworm treatment of multiple sclerosis. A total of 72 patients were enrolled, 36 were infected with 25 larvae of *Necator americanus* and 36 were given placebo. At the time of commencement, this was the largest study so far performed to assess the efficacy and safety of helminth therapy. Subsequently, the possibility that helminth therapy might induce Epstein-Barr virus (EBV) reactivation has been

raised. Reese and colleagues have reported (225) that helminth challenge/infection of mice previously infected with the murine gamma herpesvirus (MHV68) blocked the antiviral activity of interferon-γ and promoted virus replication and that in cell culture experiments reactivation of herpesvirus was induced. In a separate study using mice (130), Osborne and colleagues have shown helminth infection to have specific immunomodulatory effects on antiviral immunity. No data is available on the impact of helminth therapy on EBV reactivations in multiple sclerosis patients and because of the recently published animal data further investigation is warranted.

Other herpesviruses (eg. CMV and VZV) also widely infect humans and have the capacity to establish latency (see section 1.4). Certain disease modifying treatments (eg. fingolimod) have been associated with an increased risk of VZV reactivation (226). No data is available on how helminth treatment might impact on the humoral immunity of multiple sclerosis patients to α-herpesviruses and β-herpesviruses.

2.2 Study aim and objectives

2.2.1 Aim

To measure over a period of one-year antibody levels of selected herpes viruses and identify cases of reactivation in a group of multiple sclerosis patients therapeutically infected with *Necator americanus* and a group of placebo controlled patients recruited to the Nottingham WIRMS study.

2.2.2 Objectives

1. Characterize herpes virus antibody responses over time in *N. americanus* treated and untreated patients.
2. Determine if *N. americanus* treatment results in reactivations of α-, β, or γ- herpesviruses.
3. Investigate potential linkages between multiple sclerosis disease activity and herpesvirus antibody markers and levels.
4. Show if antibody levels are stable over time.
5. Calculate the reproducibility of the antibody detection assays used.

2.3 Study plan

Sera collected at defined time intervals during the recently completed Nottingham WIRMS clinical trial (NCT 01470521) were used for this study for which ethical approval had been obtained. The study comprised two phases; firstly, laboratory testing undertaken by PACM who had no prior access to treatment allocations (unbiased phase) or other patient clinical data. In the second phase, on completion of

laboratory testing and the documentation of results, treatment allocations and basic patient demographic data were made available to PACM, so that relevant analyses could be undertaken. The patients had monthly examinations and MRI scans between the third and ninth months of the trial and stool ova counts were measured over the period of the trial. In both cases, clinical interpretation (eg. MRI) and ova counting was conducted by individuals with no knowledge of treatment allocations.

3.0 MATERIALS AND METHODS

3.1 Equipment and materials used

3.1.1 Control sera

SERION ELISA *classic* (Institut Virion\Serion GmbH, Würzburg, Germany) control sera were obtained from Launch Diagnostics (New Ash Green, Longfield, Kent, UK) and upon receipt were stored at 6^{o}C +/– 2^{o}C. Details of control sera are shown in Table 3.

3.1.2 Deionized water

Deionized water (LAB 3 Ltd, code WA11200-275) was obtained from VWR International Ltd, Magna Park, Lutterworth, Leicestershire, UK.

3.1.3 Enzyme immunoassay kits (EIAs)

SERION ELISA *classic* (Institut Virion\Serion GmbH, Würzburg, Germany) kits were obtained from Launch Diagnostics (New Ash Green, Longfield, Kent) and upon receipt were stored at 6^{o}C +/– 2^{o}C. Details of kits are shown in Table 3.

3.1.4 Microplate reader

A BioRad Benchmark Plus microplate spectrophotometer was used to read optical densities.

3.1.5 Microplate washer

A Thermoscientific WELLWASH microplate washer (Cat no. 5165000) was used to wash microplates.

3.1.6 Rheumatoid factor

SERION ELISA *classic* (Institut Virion\Serion GmbH, Würzburg, Germany) rheumatoid factor absorbent (Z200, lot SGG.CV, expiry 2018-07) was obtained from Launch Diagnostics (New Ash Green, Longfield, Kent) and upon receipt was stored at 6ºC +/– 2ºC.

Table 3. Details of SERION ELISA *classic* kits and control sera used

ELISA kit/Control serum	Kit code	Batch ID	Expiry
Cytomegalovirus IgG ELISA	ESR109G	SHG.EN	2018-08
Cytomegalovirus IgG control serum	BC109G	SHG.BB	2018-08
Epstein-Barr virus EA IgG ELISA	ESR1363G	SHF.DP	2017-08
Epstein-Barr virus EA IgG control serum	BC1363G	SGG.GH	2018-07
Epstein-Barr virus EBNA-1 IgG ELISA	ESR1362G	SLG.CA	2018-10
Epstein-Barr virus EBNA-1 IgG control serum	BC1362G	SGG.GI	2018-07
Epstein-Barr virus EBV VCA IgG ELISA	ESR1361G	SHG.AU	2018-07
Epstein-Barr virus EBV VCA IgG control serum	BC1361G	SGG.GK	2018-07
Epstein-Barr virus EBV VCA IgM ELISA	ESR1361M	SFG.GW	2018-06
Epstein-Barr virus EBV VCA IgM control serum	BC1361M	SHG.CP	2018-08
Varicella-zoster virus IgG ELISA	ESR104G	SHG.AN	2018-06
Varicella-zoster virus IgG control serum	BC104G	SKF.BA	2017-10

3.2 Study population

The serum samples tested were collected as part of the WIRMS (Worms for Immune Regulation in MS) trial (NCT 01470521), which was conducted at the Department of Clinical Neurology, University of Nottingham. In this study, 36 patients with multiple sclerosis were treated with the hookworm *N. americanus* (25 larvae applied cutaneously per patient) and 36 received placebo (water). Patients meeting McDonald criteria for relapsing and remitting multiple sclerosis were included together with secondary multiple sclerosis patients (superimposing relapse, subject to specific clinical criteria). Adequate serum samples were available from 51 study participants. Key study timings are shown in Table 4 although it should be noted that this study was not a component of the original WIRMS study and serum samples from several patients were not available at the specified time points because they were used for other purposes or the patients had not attended the specified appointments. The available patient demographics are shown in Table 5.

Table 4. The WIRMS study: timings and key events

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**
Medical assessment and informed consent	☒														
Blood taken for laboratory investigations	☒			☒	☒	☒	☒	☒	☒	☒	☒	☒	☒	☒	☒
Randomization to helminth or placebo		☒													
MRI						☒	☒	☒	☒	☒	☒	☒	☒		☒
Mebendazole													☒		

Table 5. Patient demographics

Patient number	Age on enrolment	Sex	Treatment	
			Helminth	Placebo
3	49	M	Helminth	
6	37	F	Helminth	
7	27	F		Placebo
9	37	F		Placebo
11	42	M		Placebo
12	57	F	Helminth	
18	45	F	Helminth	
20	35	F		Placebo
22	36	F	Helminth	
25	54	F	Helminth	
27	49	F	Helminth	
28	59	F		Placebo
29	36	F	Helminth	
31	47	F		Placebo
33	48	F	Helminth	
34	32	F		Placebo
35	56	M		Placebo
36	46	F	Helminth	
37	59	M	Helminth	
38	29	F		Placebo
39	52	F	Helminth	
40	41	F		Placebo
41	32	F	Helminth	
43	39	F	Helminth	
44	62	F		Placebo
45	40	M		Placebo
46	46	F	Helminth	
47	56	F	Helminth	
48	46	F		Placebo
49	32	F		Placebo
50	46	F		Placebo
51	32	F	Helminth	
52	54	M		Placebo
53	39	F	Helminth	
54	63	F		Placebo
55	60	F	Helminth	

Patient number	Age on enrolment	Sex	Treatment	
			Helminth	Placebo
56	59	F		Placebo
57	53	F	Helminth	
58	34	F	Helminth	
59	37	F		Placebo
60	54	F		Placebo
61	64	M		Placebo
62	29	F		Placebo
64	34	F	Helminth	
65	41	M	Helminth	
66	40	F	Helminth	
68	37	M	Helminth	
69	41	F		Placebo
70	53	M	Helminth	
71	46	M		Placebo
72	54	F		Placebo

3.3 Sample collection and storage

Serum samples were collected over the period September 2012 – April 2016. Approximately, 1.5 ml blood was collected into BD Vacutainer tubes (Beckton-Dickinson) and serum separated by centrifugation at 1000-1300 relative centrifugal force for 10 minutes. Multiple aliquots of the serum samples were stored at -80^{o}C. Venepuncture and serum separation was not performed by PACM. Serum samples were tested in batches (assay runs) during which time they were stored at 2 – 8^{o}C so as to avoid multiple freeze-thawing.

3.4 Rheumatoid factor absorptions

Rheumatoid factor Z200 stock was stood at laboratory temperature for one hour and then mixed. Rheumatoid factor

containing assay VCA IgM assay dilution buffer at 20% vol/vol was prepared in test tubes by adding 200 µl rheumatoid factor (Z200) to 800 µl dilution buffer. A volume of 10 µl of serum sample was then added to each tube and the contents vortexed. The tubes were then stored overnight at 6°C +/– 2°C. They were then stood at laboratory temperature for one hour and the contents vortexed before loading onto VCA IgM assay plates.

3.5 ELISA methodology

ELISA kits were brought to laboratory temperature by standing on the bench for one hour. Serum samples were diluted 1:100 in kit-specific dilution buffer by adding 10 µl to 1,000 µl dilution buffer (see above for VCA IgM assay). Ready to use calibrators, control sera and diluted test sera were vortexed and designated antigen coated wells of each plate were loaded with 100 µl of the appropriate analyte. Well A1 was reserved as a blank and loaded with 100 µl dilution buffer, well B1 was loaded with negative control, wells C1 and D1 were loaded with standard/calibrator serum. Positive control and test sera were loaded (100µl/well) into the other test wells. The plates containing the loaded strips were placed in humid chambers and incubated for 60 min (+/- 5 min) at 37°C (+/- 1°C). During this time wash buffer was prepared by appropriately diluting the 30x wash concentrate in sterile deionized water (SDW) eg. 20ml added to 600 ml SDW. At the end of the 60 min incubation the plates were washed using the Demo 8 protocol (wash vol. = 300µl/well; no. of washes = 3) of the microplate washer. After the final wash, the plates were tapped against a paper towel (to remove

any residual wash buffer) and conjugate added, at 100µl/well, using a multichannel pipette. The plates were then incubated in humid chambers for 30 min (+/- 1 min) at 37°C (+/- 1°C). Conjugate was removed by washing using the Demo8 protocol and substrate added to all wells at 100µl/well, using a multichannel pipette. The plates were then incubated in humid chambers for a further 30 min (+/- 1 min) at 37°C (+/- 1°C). The substrate reaction was then stopped by addition of 100 µl stopping solution to all wells using a multichannel pipette. The optical densities (ODs) of the reactions were then read, within 20 min, using a BioRad microplate reader. The reader was set at 405 nm with a reference wavelength of 650 nm.

3.6 Interpretation and quantification of ELISA results

The recorded optical densities were modified by subtraction of the blank $OD_{405/650}$ from the test $OD_{405/650}$s. The assay results were then validated against the kit-specific validation criteria specified on the manufacturer's quality control certificates. Subject to passing the validation criteria the results were interpreted qualitatively with reference to lot specific data supplied on the manufacturer's quality control certificate. A positive test result confirms that specific antibodies have been detected. For EBV, a panel of antibody results (Table 6) has to be interpreted so that the stage of infection (acute/recent, past and reactivation) can be assigned.

Table 6. SERION ELISA *classic* guidelines for the interpretation* of EBV serology in immunocompetent individuals.

Interpretation	EBV assay result profile			
	EA IgG	VCA IgM	VCA IgG	EBNA-1 IgG
Not infected or pre-seroconversion	Neg	Neg	Neg	Neg
Acute/recent infection	Pos	Neg	Neg	Neg
	Neg	Pos	Neg	Neg
	Pos	Pos	Neg	Neg
	Pos	Pos	Pos	Neg
	Pos	Neg	Pos	Neg
	Neg	Pos	Pos	Neg
	Neg	Neg	Pos	Neg
Past infection	Neg	Neg	Pos	Pos
	Neg	Neg	Neg	Pos
Reactivation	Pos++[1]	Neg	Pos++	Pos
	Pos++	Pos	Pos++	Pos

*Cut-off criteria of "Neg" (Negative) and "Pos" (Positive) specified in manufacturer's batch specific kit insert. [1]Pos++ notation used by manufacturer and assumed to be high antibody level, but no defined level stipulated.

3.7 Quantification of assay results

Modified antibody ODs (blank subtracted) can also be used to generate quantitative results due to the incorporation of standard serum in each assay run and the provision of a kit, lot-specific standard curve (4 parameter, logistic) by the manufacturer. The standard serum acts as a single point calibrator and the calculation of a correction factor (F = the manufacturer's assigned standard OD value/mean assay measured run value) enables correction for variation of test OD run values in any particular assay run so that they can be interpolated directly from the supplied standard curve. Correction is attained by multiplying the assay run test OD values by the calculated correction factor (F).

3.8 Quality assurance of assay reproducibility

The inclusion of an independent positive control serum in every assay run allows within run variation and between run variation to be calculated. In every test run, positive control serum was loaded into wells E1, F1 and H6 (in some runs G6 loaded, as well). The positive control sera have an assigned target value and target range so assay run data can be validated.

3.9 Statistical Methods

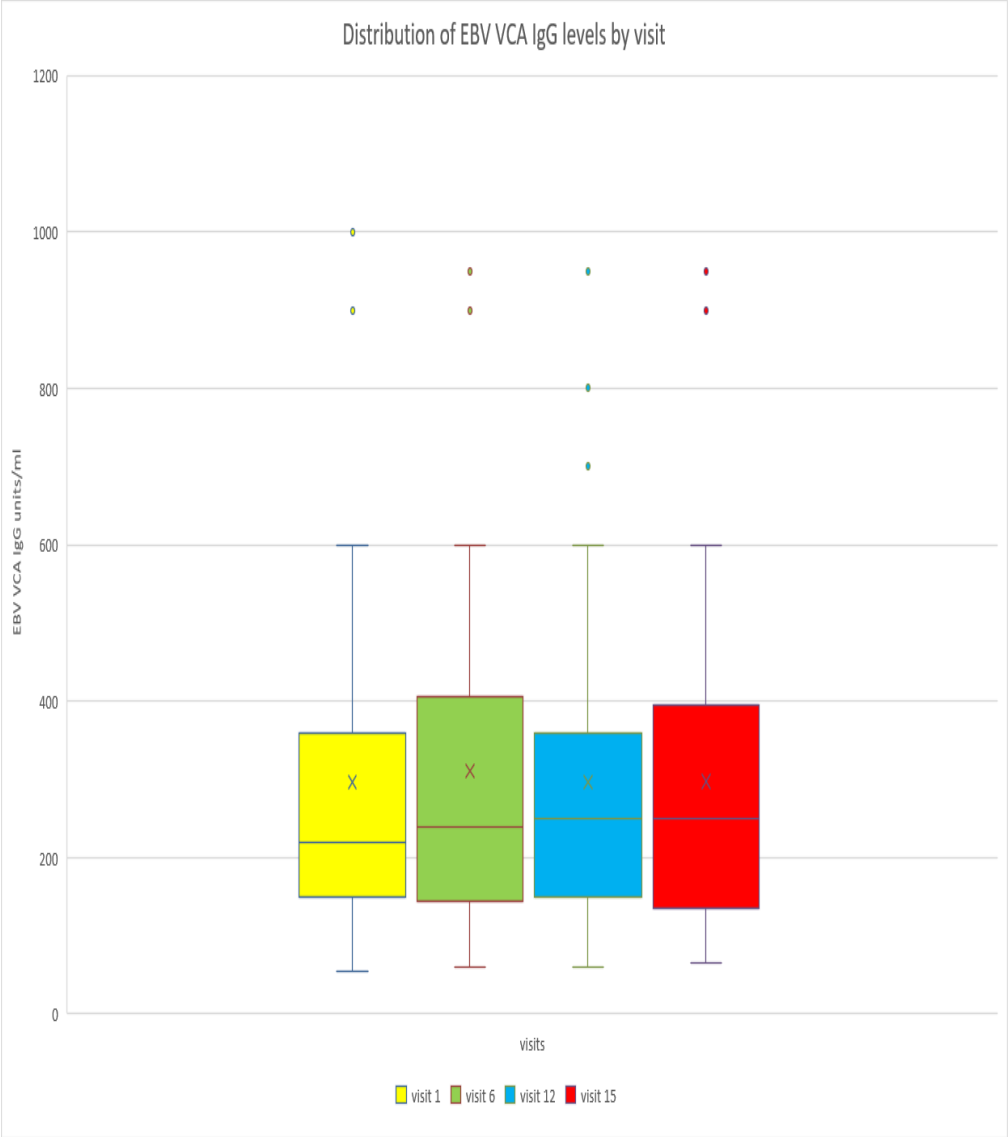
Basic data manipulations; for example, calculation of means and standard deviations together with graphical presentations were performed using Excel 2016. Statistical analysis was performed using Prism 7 software and significance was set at $p < 0.05$. Details of the statistical tests applied are given in the text. Associations of categorical data (eg. sero-prevalence) were tested using Fisher's exact test because of the small sample sizes available. Associations of non-paired, continuous data (eg. geometric means) were tested using an independent samples t-test and a normal distribution within groups was assumed.

RESULTS

4.1 Qualitative and quantitative EBV antibody levels in helminth treated (n = 26) and placebo controlled patients (n = 25).

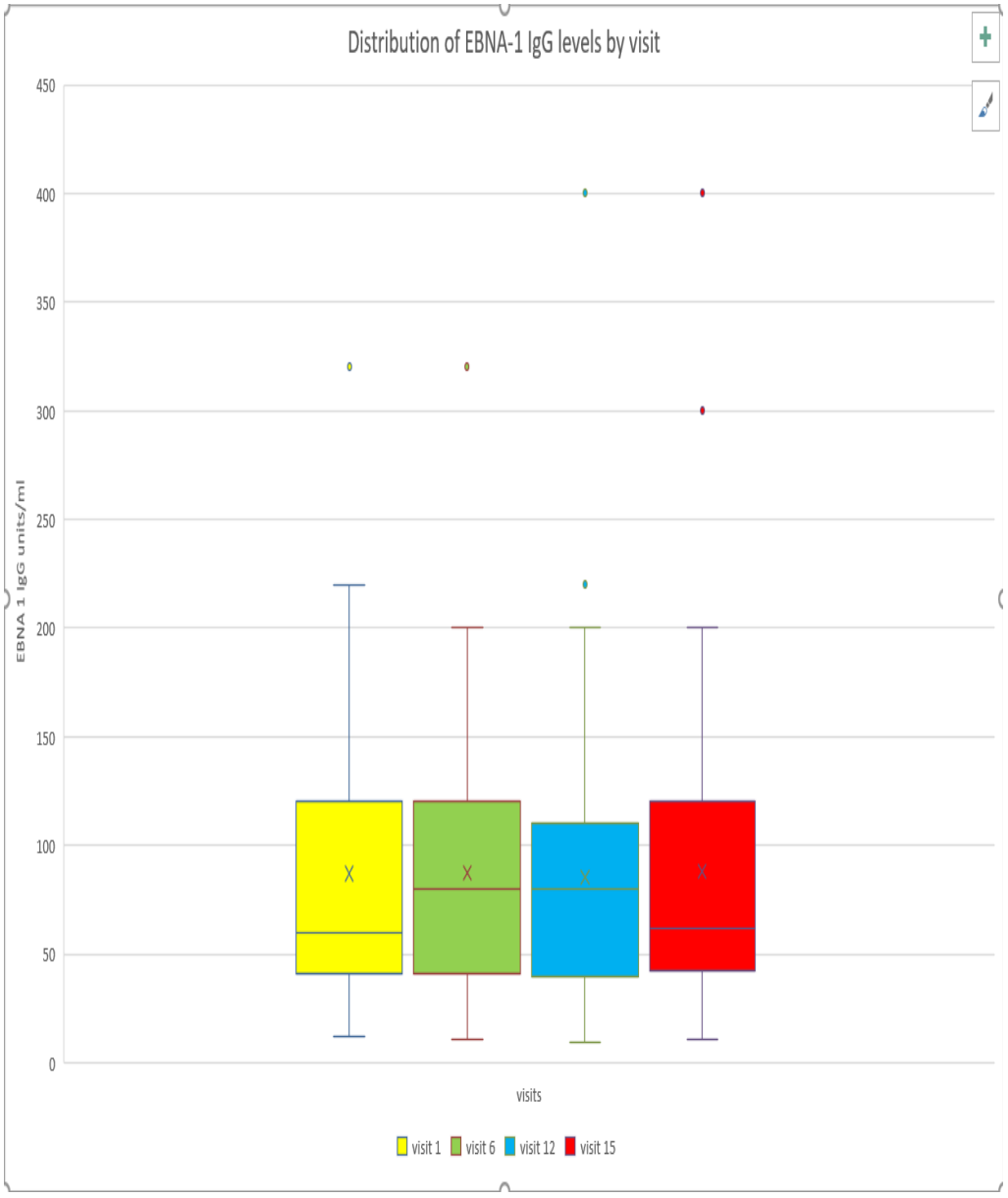
All helminth treated, and placebo controlled multiple sclerosis patients tested positive for EBV viral capsid IgG (VCA IgG) and nuclear antigen-1 IgG (EBNA-1 IgG). The antibody levels were very stable over the one-year period of the study (Figures 2a and 2b). A total of 8 (30.7%) helminth treated patients were EBV early antigen (EA) positive compared to 10 (40.0%) placebo controlled patients. The difference in the EBV EA IgG sero-positivity between the two groups was not significant (Fisher exact test statistic value = 0.565). There were a number of equivocals in both groups and these have been treated as sero-negatives. It should also be noted that there was some variation in the cut-off values between assay runs and these are shown in Table 7.

Figure 2a. Distribution of EBV VCA IgG levels in all patients (n = 51) tested



Box and whisker plot also showing outliers (o) of EBV VCA IgG levels of all patients tested for whom sequential sera were available collected at visits 1, 6, 12 or 13, and 15. The edges of the box moving upwards represent 25th and 75th percentiles and the internal line the exclusive median. The mean is represented by (x).

Figure 2b. Distribution of EBNA-1 IgG levels in all patients (n = 51) tested



Box and whisker plot also showing outliers (o) of EBNA-1 IgG levels of all patients tested for whom sequential sera were available collected at visits 1, 6, 12 or 13, and 15. The edges of the box moving upwards represent 25th and 75th percentiles and the internal line the exclusive median. The mean is represented by (x).

Table 7. Variation of cut-off values for EBV EA IgG with assay run

Assay run	Mean $OD_{405/650}$	Manufacturer's stipulated cut-offs $OD_{405/650}$		
	Standard serum	Pos cut-off	Equiv range	Neg cut-off
1	0.89	>0.27	0.18 - 0.27	<0.18
2	0.88	>0.27	0.18 – 0.27	<0.18
3	1.0	>0.31	0.21 - 0.31	<0.21
4	0.95	>0.29	0.20 - 0.29	<0.20
5	0.86	>0.25	0.17 - 0.25	<0.17
6	0.89	>0.27	0.18 – 0.27	<0.18

The detection of EBV EA IgG in the presence of EBV VCA IgG and EBNA-1 IgG is potentially consistent with virus reactivation (see Table 6) so approximately 30% - 40% of patients tested showed evidence of EBV reactivation. In all these cases, the pre-treatment serum was EBV EA IgG positive and there was no evidence of a trend of increasing EA IgG levels during treatment. Another marker of EBV reactivation is the detection of EBV VCA IgM in patients with evidence of past EBV infection (ie. EBNA-1 IgG positive). One helminth treated patient (no. 36) was EBV VCA IgM positive and one placebo-controlled patient (no. 48) was EBV VCA IgM positive. In both cases, the pre-treatment serum samples were EBV VCA IgM positive and there was no significant trend of increasing IgM levels during treatment.

Interpolation from the standard curves generated for each assay run enabled quantitative antibody levels to be determined. The

manufacturer's stated limits of quantification for the EBV VCA IgG assay were 5 - 200 units/ml (U/ml) and the serum levels detected in the multiple sclerosis patients studied ranged from 55 U/ml to >200 U/ml. In the helminth treated group, 78.8% serum VCA IgG levels measured >200 U/ml and in the placebo controlled group 58.4% of serum VCA IgG levels measured >200 units/ml. A total of 20/26 (76.9%) patients in the helminth treated group and 14/25 (58.4%) patients in the placebo controlled group commenced the study with EBV VCA IgG levels >200 units/ml and this difference was not significant (Fisher exact test value 0.144). By imputing a level of 201 units/ml to values >200 units/ml, geometric mean levels of 182.9 units/ml and 163.5 units/ml were generated for helminth treated and placebo controlled groups, respectively. Several sera with EBV VCA IgG levels >200 units/ml were subsequently titrated out (see Figure 2a).

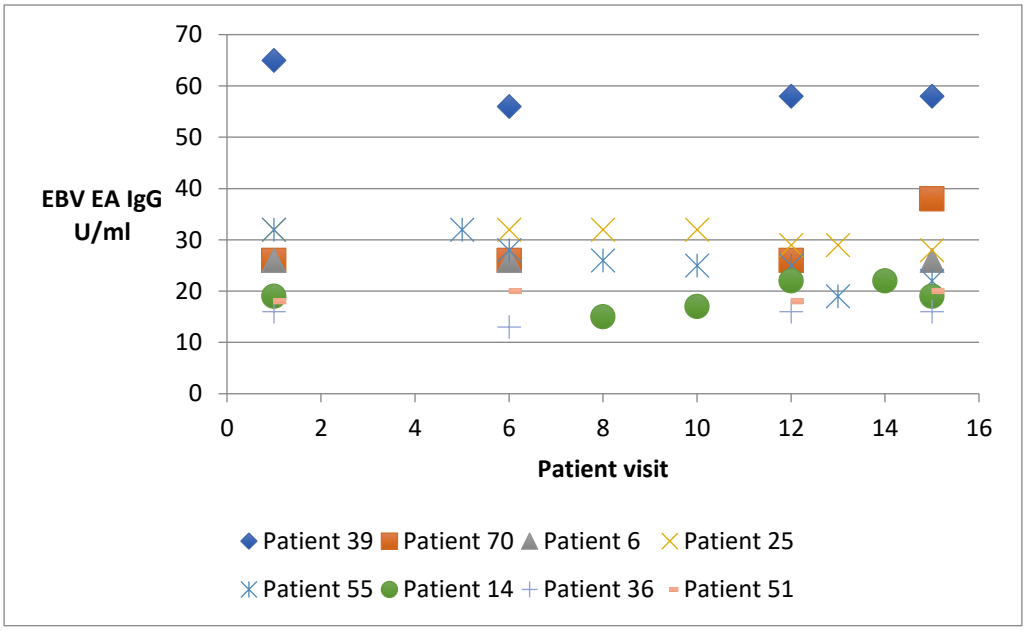
For the EBNA-1 IgG assay the manufacturer's stated limits of quantification were 1 – 200 U/ml and the serum levels detected in the multiple sclerosis patients studied ranged from 9.5 U/ml to >200 U/ml. In the helminth treated group 1.7% serum EBNA-1 IgG levels measured >200 U/ml and in the placebo controlled group none of the serum EBNA-1 IgG levels measured >200 U/ml. By imputing a level of 201 U/ml to values >200 U/ml, geometric mean levels of 68.3 U/ml and 68.5 U/ml were generated for the helminth treated and placebo controlled groups, respectively. The EBNA-1 IgG levels in helminth treated and placebo controlled groups were not statistically different (Independent samples t-test, t-value = 0.201, p-value = 0.42). Several sera with

EBNA-1 IgG levels >200 units/ml were subsequently titrated out (see Figure 2b).

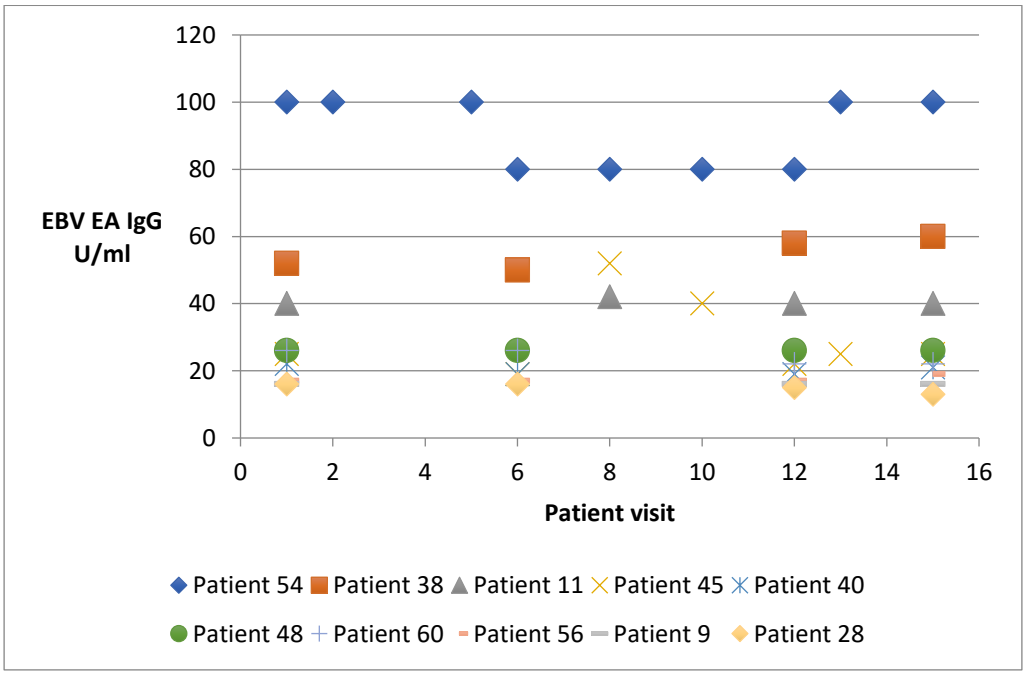
The manufacturer's stated limits of quantification for the EBV EA IgG assay were 2 - 400 units/ml (U/ml) and the negative cut-off was <10 U/ml, equivocal range was 10 U/ml – 15 U/ml, and positive cut-off was >15 U/ml. In the helminth treated group using the aforementioned quantitative cut-offs, 14 patients were EA IgG negative, 3 were equivocal, and 8 were EA IgG positive (range 16 U/ml – 65 U/ml). In the placebo controlled group, 12 patients were EA IgG negative, 3 were equivocal, and 10 were EA IgG positive (range 16 U/ml – 100 U/ml). Generally, EA IgG levels were stable over time, but some patients showed more variation than others (Figure 3). Only one patient (patient no 36) in the helminth treated group was EBV VCA IgM positive and levels over the period of the study ranged between 40 – 70 U/ml, mean 52.7 U/ml. Similarly, only one patient (patient no 48) in the placebo controlled group was EBV VCA IgM positive and levels over the period of the study ranged between 25 – 30 U/ml, mean 27 U/ml. Both patients 36 and 48 were EA IgG positive with levels ranging over the period of the study of 13 – 16 U/ml and 26 U/ml, respectively.

Figure 3. EBV early antigen levels over time in (a) helminth treated, and (b) placebo controlled patients

(a) Helminth treated patients



(b) Placebo controlled patients



4.2 Qualitative and quantitative CMV and VZV antibody levels in helminth treated (n = 26) and placebo controlled patients (n = 25).

A total of 13 (50%) helminth treated patients and 4 (16.0%) placebo control patients were positive for CMV IgG. The difference in the CMV IgG sero-positivity between the two groups was significant (Fisher exact test value = 0.016). Generally, the CMV IgG levels were stable over the one-year period of the study (Figure 4a); however, in one CMV IgG negative patient (no. 45) there was a transient seroconversion at visit 8. The visit 8 test result was reproducible and on further investigation of clinical history (eg. administration of blood products) there was no explanation for this finding. At visit 10, two months later, the patient had reverted to being CMV IgG negative. All samples tested were VZV IgG positive and the antibody levels detected were very stable over the period of the study (Figure 4b) even in the patient (no. 45) with the transient CMV IgG seroconversion (VZV IgG $OD_{405/650}$s were 1.86, 1.86 and 1.87 at visits 6, 8, and 10, respectively). The absence of a spike in VZV IgG levels at visit 8 for patient no. 45 would mitigate against the CMV IgG spike at the same visit been a false-positive reaction and measurement of CMV DNA may have proved informative; however, it was not possible to undertake this investigation.

The manufacturer's stated limits of quantification for the CMV IgG assay were 10 - 2000 PEI (Paul Ehrlich Institute) units/ml (U/ml) and the negative cut-off was <25 PEI-U/ml, equivocal range was 25

PEI-U/ml – 40 PEI-U/ml, and positive cut-off was >40 PEI-U/ml. In the helminth treated group using these cut-offs, 13 patients were CMV IgG negative and 13 were CMV IgG positive (range 120 PEI-U/ml – 1700 PEI-U/ml; geometric mean = 428 PEI-U/ml). In the placebo controlled group, 21 patients were CMV IgG negative and 4 were CMV IgG positive (range 85 PEI-U/ml – 1400 PEI-U/ml; geometric mean = 321 PEI-U/ml). Generally, CMV IgG levels were stable over time; however, in one patient (no. 45) CMV IgG levels were <10 PEI-U/ml at visits 1 and 6, then increased to 750 PEI-U/ml at visit 8, and reverted to <10 PEI-U/ml at visits 10, 12, 13 and 15.

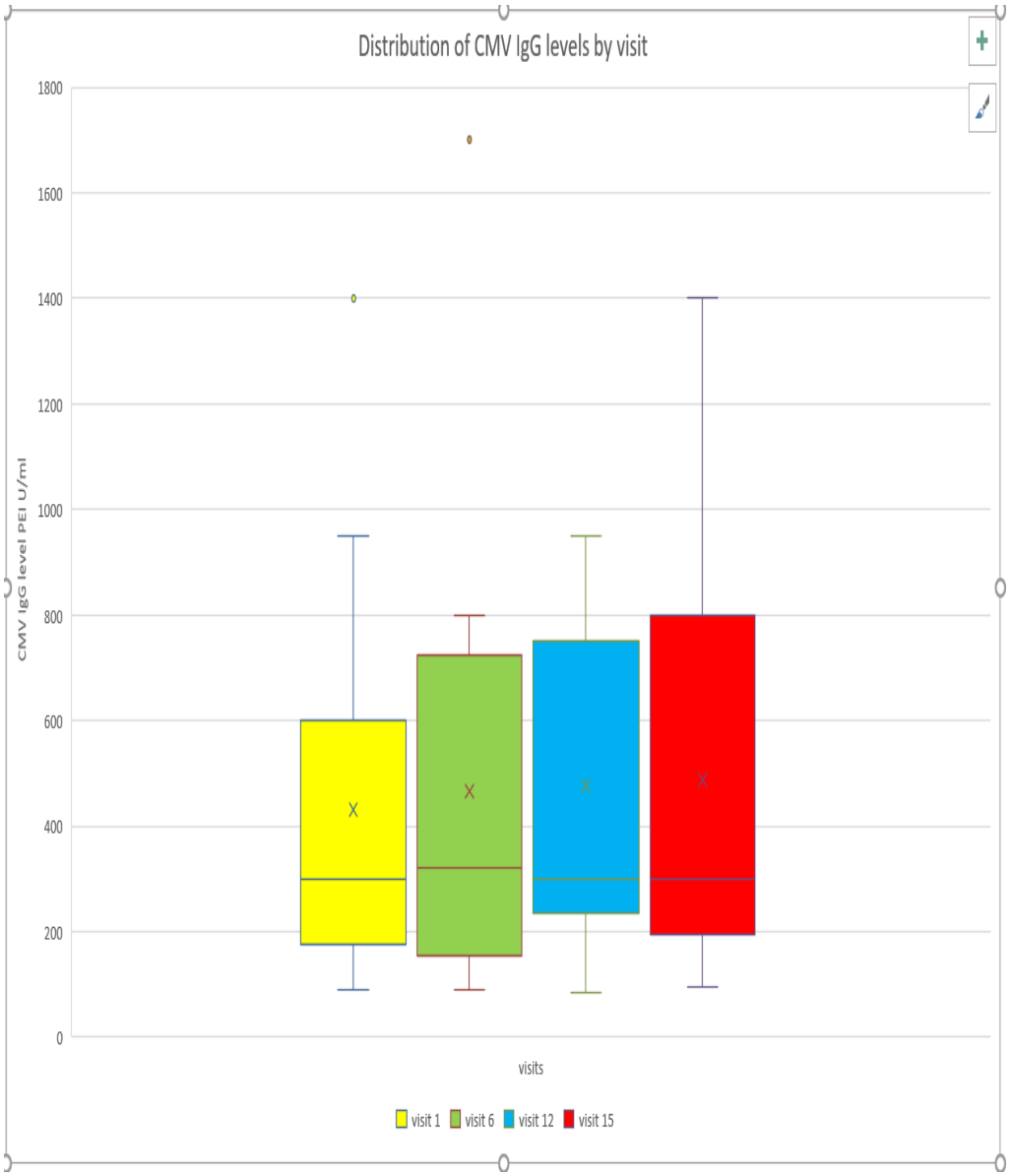
For the VZV IgG EIA the manufacturer's stated limits of quantification were 15 – 2000 mIU/ml and the negative cut-off was <50 mIU/ml, equivocal range was 50 mIU/ml – 100 mIU/ml, and positive cut-off was >100 mIU/ml. The range of VZV IgG levels detected was 220 mIU/ml - >2000 mIU/ml in both the helminth treated and placebo controlled groups. By imputing a level of 2001 mIU/ml to values >2000 mIU/ml, geometric mean levels of 1098 mIU/ml and 1200 mIU/ml were generated for the helminth treated and placebo controlled groups, respectively. The VZV IgG levels in helminth treated and placebo controlled groups were statistically different (Independent samples t-test, t-value = -2.27, p-value = 0.011). Several sera with VZV IgG levels >2000 mIU/ml were subsequently titrated out (see Figure 4b).

Table 8 provides a summary of sero-prevalence findings for the virus markers tested.

Table 8. Summary of sero-prevalence findings for helminth treated (n = 26) and placebo controlled (n = 25) patients

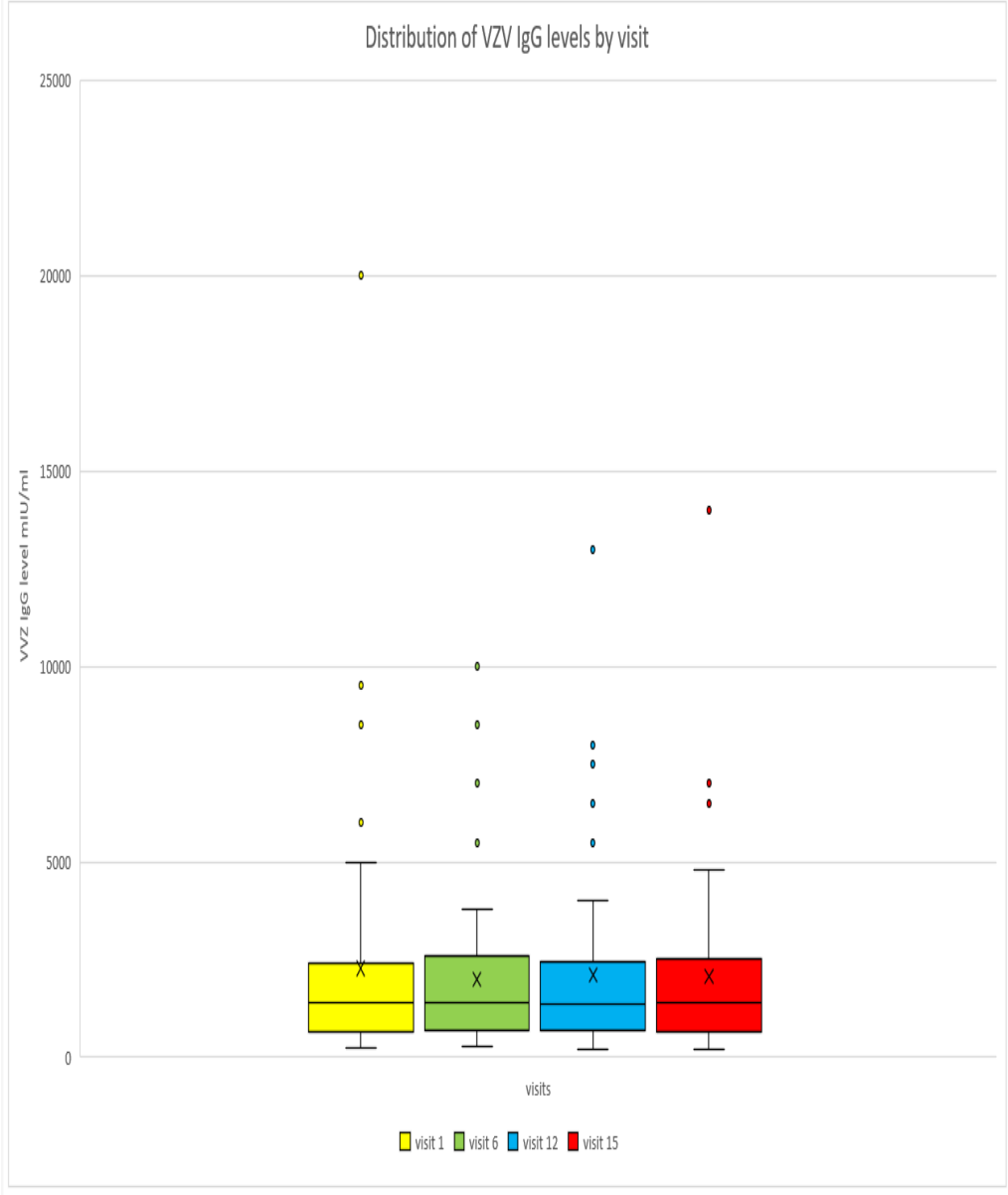
Virus marker	Rate of antibody positives (%)	
	Helminth treated	Placebo controlled
EBV VCA IgG	100%	100%
EBV VCA IgM	3.8%	4.0%
EBNA-1 IgG	100%	100%
EBV EA IgG	30.7%	40%
CMV IgG	50%	16%
VZV IgG	100%	100%

Figure 4a. Distribution of CMV IgG levels in all patients (n = 51) tested



Box and whisker plot also showing outliers (o) of CMV IgG levels of all patients tested for whom sequential sera were available collected at visits 1, 6, 12 or 13, and 15. The edges of the box moving upwards represent 25th and 75th percentiles and the internal line the exclusive median. The mean is represented by (x).

Figure 4b. Distribution of VZV IgG levels in all patients (n = 51) tested



Box and whisker plot also showing outliers (o) of VZV IgG levels of all patients tested for whom sequential sera were available collected at visits 1, 6, 12 or 13, and 15. The edges of the box moving upwards represent 25th and 75th percentiles and the internal line the exclusive median. The mean is represented by (x).

4.3 Determination of assay variability

4.3.1 Within run variability

In all assay runs (Table 9) there was an average coefficient of variation (COV) of 4.62% (range 0% - 12.3%) in within plate internal quality control serum quantitative antibody levels.

Table 9. Within (intra) assay coefficient of variation (%) of internal quality control serum levels

Assay run	EA IgG BC1363G %COV/run	VCA IgG BC1361G %COV/run	VCA IgM BC1361M %COV/run	EBNA-1 IgG BC1362G %COV/run	CMV IgG BC109G %COVrun	VZV IgG BC104G %COV/run
1	0%	0%	6.3%	2.06%	5.8%	10.9%
2	0%	3.49%	8.2%	3.7%	3.7%	10.4%
3	7.9%	12.0%	12.35%	3.9%	0%	7.18%
4	3.48%	0%	3.17%	10.1%	10.5%	2.98%
5	10.8%	0%	8.24%	3.27%	3.38%	0%
6	3.92%	0%	8.66%	0%	0%	0%

4.3.2 Between run variability

For the different assays used, the between run COV was no more than 12.0% (range 3.71% - 12.0%; mean = 6.35%) as determined from the mean quantitative values/run of the internal quality control sera (Table 10).

Table 10. Mean internal quality control serum levels per assay run

Assay run	EA IgG BC1363G Mean U/ml	VCA IgG BC1361G Mean U/ml	VCA IgM BC1361M Mean U/ml	EBNA-1 IgG BC1362G Mean U/ml	CMV IgG BC109G Mean PEI-U/ml	VZV IgG BC104G Mean mIU/ml
1	32	80	66	27.6	150	314
2	32	82.5	70	31	155	330
3	31.6	86.6	56	29.3	140	320
4	33	80	78.7	29.5	142.5	335
5	32	100	70	30.5	147.5	300
6	29.3	100	66.6	30	150	320

DISCUSSION

5.1 Putting the results in context

5.1.1 Defining the patient cohort

A major determinant of the outcome of any clinical trial is the patient population studied (227). In particular, the clinical phenotypes recruited may differ significantly in underlying pathology and the capacity to respond to treatment. These issues are highly relevant for multiple sclerosis and in 1996 standardized definitions for the most common clinical courses of patients with multiple sclerosis were published (228). In total, four multiple sclerosis clinical courses were identified – relapsing-remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS), and progressive-relapsing MS (PRMS). These standards were revised in 2013 (229) and although the broad classifications remain there is more emphasis on assessing disease activity and application of imaging together with relevant laboratory data. In the WIRMS study, which commenced 2012 the majority of patients were RRMS with some SPMS; however, for the purposes of the (our) study reported here the breakdown by clinical phenotype is not yet available.

5.1.2 How does helminth therapy impact upon the mechanism of multiple sclerosis and is there a rationale for predicting herpes virus reactivation?

The mechanism of disease in RRMS is predominantly inflammatory with the production of focal inflammatory lesions

characterized by perivenular accumulation of lymphocytes, disruption of the blood-brain barrier, compromise of regulatory cell activity, demyelination, and acute axonal transection (22, 230). In progressive multiple sclerosis (PMS) there is a mechanistic shift from focal inflammatory pathology towards more diffuse immunological events and domination by incompletely understood neurodegenerative mechanisms with the consequence that many treatments for RRMS have little efficacy on PMS (95, 231).

Until recently, the view prevailed that multiple sclerosis was primarily an autoimmune disease driven by T cell dysfunction (45, 232). For instance, evidence has been presented in mice that following EAE induction the immune response is dominated by interferon-γ producing T_H1 cells capable of inducing EAE when transferred to syngeneic recipients (233). More recently, the classical separation (234) of T_H cell populations into type 1 and 2 populations has been superseded by recognition of the existence of another T_H cell population – T_H17 (235). Just as γ-interferon is the signature effector cytokine of T_H1 lymphocytes, interleukin 17A (IL-17A) is the signature effector cytokine of T_H17 lymphocytes. A significant function of IL-17A is the induction of colony stimulating factors (eg. GM-CSF). Neutralization of IL-17, or IL-17 deficiency, in mice have been shown to manifest resistance to the induction of EAE (236, 237). Experimental studies have shown that T_H17 lymphocytes display considerable plasticity dependent on the inflammatory environment and that the nature of the interplay between T_H1, T_H17 and other T_H lymphocyte populations remains to be fully

determined (238). For instance, GM-CSF may be produced by T_H1 or T_H17 populations or other independent populations of T_H cells which are neither T_H1 or T_H17.

Regulatory T cells (T_{regs}) may be thymically derived (natural T_{reg} - nT_{reg}) or peripherally generated (adaptive/inducible T_{reg} - iT_{reg}) and a variety of population subsets are to be found all with the transcriptional regulator Forkhead box protein 3 (FoxP3). A key function of T_{regs} is that they act as a critical control point for the maintenance of self-tolerance; however, they possess other important functions such as the control of inflammatory processes (239). There is abundant evidence that T_{regs} modulate CNS inflammation and play a key role in the development and progression of autoimmune diseases (240). It is now well established that defective function of T_{regs} is a component of RRMS as evidenced by reduced suppressive capacity compared to healthy controls (241). The reduction of T_{regs} suppressive capacity in RRMS patients has been shown to be reversible following treatment with interferon β1a (242).

The presence of oligoclonal banding in the CSF is highly associated with multiple sclerosis and has been used as a biomarker for the disease (243). The presence of such bands in CSF reflects intrathecal oligoclonal IgG synthesis; however, this may be non-specific for multiple sclerosis (244). B lymphocytes play a major role at the site of inflammation through the release of cytokines and B regulatory cell function is important in determining the extent of pro-inflammatory activity (245). The use of B cell depletion by treatment with B cell

specific depleting monoclonals eg. rituximab) has been shown to be beneficial in the treatment of multiple sclerosis (246). There is a considerable need to gain greater knowledge of the role of effector and regulatory B cells in multiple sclerosis (247) and a recent finding of interest is that B cells from patients with multiple sclerosis induce, cytokine independent, apoptotic pathways resulting in neuron cytotoxicity (248).

It is highly evident that a diversity of immune cell types and processes contribute to multiple sclerosis (249) and it cannot be considered solely as a disease mediated by T_H1 mechanisms. The same can be said of the immune response to helminth infections in that they do not just elicit T_H2 responses (126). There is significant evidence, which has been reviewed in section 1.3.3 for the general applicability of helminth therapy in multiple sclerosis. In the context of the WIRMS (our) study there are very limited external data on how the therapeutic dose of helminth used will impact on relevant immunological pathways. If there is an effect, it remains to be determined over what time it may generate, and if it is compensated for by modification, or plasticity, of affected immune pathways. Consequently, there is a very limited database, which can be used to predict an effect of helminth therapy on herpes virus reactivation as a result of the specific conditions used in the WIRMS study. The findings reported in our study of a UK population may not be translatable to, for example, populations with natural helminth infection.

5.2 Assessment of the herpes virus antibody levels detected

5.2.1 The significance of the EBV virus antibody levels detected

All patients (n = 51) tested showed evidence of past EBV infection and this confirms previous reports of virtually 100% sero-prevalence of EBV VCA IgG and EBNA-1 IgG in patients with multiple sclerosis (181, 188). No significant difference was found between the rate (30.7%) of EBV EA IgG sero-positivity in helminth treated patients (n = 26) compared to the rate (40.0%) of EBV EA IgG sero-positivity in placebo controlled patients (n = 25). Previous studies (250) have reported similar rates (36% versus 41%) of EBV EA IgG sero-positivity in multiple sclerosis patients compared to healthy controls. Other studies (189, 199) have reported an increased sero-prevalence of EBV EA IgG in patients with multiple sclerosis. The detection of EBV EA IgG is associated with acute infection or virus reactivation; however, care is needed in making either inference as EBV EA IgG is also detected in 20% – 30% healthy controls and can persist for years after a primary infection (251). In all our cases, the pre-treatment serum was EBV EA IgG positive and there was no evidence of a trend of increasing EA IgG levels during treatment. The detection of EBV VCA IgM is principally associated with primary infection (172) and it is infrequently detected in cases of reactivation (252). EBV VCA IgM was detected in only one helminth treated patient and a placebo controlled patient. In both cases, the pre-treatment serum samples were EBV VCA IgM positive and there was no significant trend of increasing IgM levels during treatment. These data lead to the conclusion that helminth treatment

under the conditions of the WIRMS study was not associated with EBV reactivation. A significant number of patients (30% - 40%) appeared to be in a state of persistent EBV reactivation and this finding has been reported in other studies (195, 253).

There have been conflicting reports linking clinical relapse or disease progression with EBV infection/reactivation. For instance, Farrell and colleagues (190) who investigated 100 subjects over a five-year period suggested, on the basis of elevated EBNA-1 IgG titres, that there was an association between EBV infection and multiple sclerosis disease activity. Unfortunately, EBV EA IgG levels were not examined in this study and the variability of the commercial assay used for EBNA-1 IgG quantitation was not given. In our study, no significant difference in EBNA-1 IgG levels was found between helminth-treated and placebo-controlled groups. Limitations of both studies are that the EBNA-1 IgG levels obtained are not comparable and EBNA-1 IgG levels were not determined in appropriate populations to determine “normal” ranges. In another study, by Buljevac and colleagues (195), 73 RRMS patients were followed for an average of 1.7 years and no association was found between increased clinical disease activity and serological evidence of EBV reactivation. There are similarities in the findings of this study and our study; for example, EBV EA IgG levels were stable over time and EBV VCA IgM was detected in only three cases. At the time of submission an analysis of clinical disease activity in the WIRMS study patients is not available so no comment can be

made regarding clinical disease activity and the EBV antibody levels detected in our study.

5.2.2 The significance of the CMV and VZV virus antibody levels detected

A total of 13 (50%) helminth treated patients (n = 26) and 4 (16.0%) placebo controlled patients (n = 25) were positive for CMV IgG. The difference in the CMV IgG sero-positivity between the two groups was significant (Fisher exact test value = 0.016). Generally, the CMV IgG levels were stable over the one-year period of the study. The disparity between CMV IgG sero-prevalence in helminth treated patients compared to placebo controlled patients raises the question of recruitment bias due to unknown factors. In studies (195) in which CMV IgG seroprevalence has been compared between multiple sclerosis patients and healthy controls no disparity has been observed. In an urban region of the UK, significant disparities in CMV IgG sero-prevalence have been observed between women of different ethnicity (254) and this may be a factor contributing to the disparity of CMV IgG sero-prevalence between groups observed in our study. Population CMV IgG sero-prevalences of 41.5% - 76.7% have been reported in Western European countries (255) so the detected sero-prevalence of 16.0% in our placebo controlled patient group is highly atypical.

There have been conflicting reports regarding the significance of CMV infection in multiple sclerosis patients. Horakova and colleagues (256) have reported that CMV positivity increases the likelihood of

relapses in clinically isolated syndrome (CIS) patients. The same group has also shown that humoral immunity to CMV is associated with brain atrophy in CIS patients treated with interferon-β (218). It has been suggested (219) that cytomegalovirus infection causes chronic activation of the immune system and that cytotoxic and pro-inflammatory $CD4^{+}CD28^{null}$T cells are only present in CMV seropositive candidates, which promote autoimmune mediated neuroinflammation. Conversely, there have been other reports that attribute to CMV positivity a disease-limiting role (257) or negative association with the risk of disease manifestation (217). Several mechanisms have been proposed for this variety of effects of CMV infection in relation to multiple sclerosis (258).

In our study, all patients tested (n = 51) were VZV IgG positive and there was a statistically significant difference in antibody levels between helminth treated (geometric mean = 1098 mIU/ml) and placebo controlled groups (geometric mean = 1200 mIU/ml). There have been reports (259, 260) of higher rates of VZV IgG sero-prevalence or prior varicella/herpes zoster in patients with multiple sclerosis compared to the general population. Unfortunately, in our study a control population has not been tested so no comparisons of VZV IgG sero-prevalence/antibody levels can be made. In the laboratory, VZV reactivation (shingles) is best determined by detecting a significant increase in VZV IgG levels or through viral load measurements (261) as detection of VZV IgM is unreliable due to the lack of sensitivity of routinely available assays (262, 263). In our study,

no serological evidence of VZV reactivation was seen in any of the patients tested. The apparent difference in VZV IgG levels between helminth treated and placebo controlled patients may reflect an unknown selection bias and when the WIRMS study clinical data is fully available can be explored further.

5.3 Conclusions

In this study, the herpes virus antibody levels (EBV EBNA-1 IgG, EBV VCA IgG, EBV VCA IgM, EBV EA IgG, CMV IgG, VZV IgG) in helminth treated and placebo controlled multiple sclerosis patients have been monitored over a period of time with the following findings.

1. All individuals tested (n = 51) showed evidence of past EBV infection.

2. Compared to animal studies (130, 225) no evidence of EBV reactivation in humans therapeutically infected with a low number of helminth ova has been found.

3. Over a period of one-year EBNA-1 IgG, EBV VCA IgG, and EBV VCA IgM antibody levels were stable.

4. EBV EA IgG was detected in 30.7% helminth treated patients (n = 26) compared to 40.0% placebo controlled patients (n = 25). The difference was not significant ($p<0.05$). Levels were stable throughout the period of the study including pre-treatment and post-treatment phases. The significance of this finding warrants confirmation by further investigations.

5. CMV IgG and VZV IgG levels were stable throughout the period of the study and there was no serological evidence of CMV or VZV reactivation.

6. CMV IgG was detected in 50% helminth treated compared to 16.0% placebo controlled patients. This difference was significant ($p<0.05$) and raises the issue of recruitment bias due to unknown factors.

7. VZV IgG was detected in all patients tested and there was a significant ($p<0.05$) difference in levels between helminth treated and placebo controlled groups. The antibody levels were very stable throughout the period of sampling, so the disparity represents potential recruitment bias.

8. Vaccines are either available (eg. VZV) or under development (CMV, EBV) for the human herpesviruses tested in our study. Likewise, a number of new therapeutic agents (eg. alemtuzumab) are entering clinical use for which herpes virus reactivations have been recorded. Our findings will help inform their current or future use.

5.4 Future work

The collection of samples from the WIRMS study with associated clinical data represents a high-value, scientific resource. Because sera were multiple aliquotted at the time of sample processing there is the facility to reduce freeze-thawing episodes of samples to a minimum. Some additional laboratory testing could help resolve several issues raised in the Discussion, as follows.

1. Testing a control group of samples from a “general” or non-multiple sclerosis cohort using the same methodology would enable comparative sero-prevalences of herpes virus antibodies to be determined. Such data would either support or refute previously published findings of associations of herpes virus infections with the development of multiple sclerosis or aspects of multiple sclerosis such as disease severity.

2. Full quantitation of antibody levels by testing higher dilutions of sera to determine quantitative end-points would enable valid comparisons of quantitative antibody levels to be made with clinical or radiologically determined disease activity.

3. The utility of EBV VCA EA IgG detection as a marker of virus reactivation needs to be assessed further. Do patients with evidence of persistent EBV reactivation by this assay have adverse clinical outcomes compared to controls? Confirmatory serological testing and viral load data would be useful for this group.

4. The findings following CMV IgG testing are remarkable. A greater understanding of the role of CMV infection is much needed, as there is the possibility of therapeutic intervention, if necessary. In view of the conflicting findings from published reports further studies at the cellular level are required to increase our understanding of the interactions of the immune systems of multiple sclerosis patients at different stages of disease with this virus.

5. Sero-prevalence and quantitative antibody levels should be determined for the other human herpes viruses (eg HHV-1/2 and HHV-6/7) not tested in this study. Herpes Simplex viruses 1 and 2 (HHV-1/2) are very closely related herpesviruses belonging to the genus *Simplexvirus* and like VZV are classified in the subfamily *Alphaherpesvirinae* (264). Similar to VZV, HHV1/2 viruses establish latency in sensory neurons; however, they adopt very different modes of pathogenesis and have significantly different clinical manifestations and aetiologies (265). Historically, HHV-1 infection was associated with herpes gingivostomatitis and HHV-2 infection was associated with genital herpes, but these boundaries are now blurred as a consequence of changes in sexual behaviour. Both HHV-1/2 are significant causes of neurological infections and show different characteristic clinical presentations (266). HHV-1 is a major cause of sporadic encephalitis in immunocompetent individuals while HHV-2 is associated with aseptic meningitis in adults. In immunosuppressed patients and the neonate, infection by either virus can disseminate with significant morbidity and mortality. Diagnosis of infection is by nucleic

acid detection of virus DNA following polymerase chain reaction using appropriate samples. For suspected encephalitis, it is recommended that serum and CSF samples be analysed – CSF should be tested for virus DNA and both samples tested for antibody to determine intrathecal production (267). Unfortunately, for this study serum samples only were available for testing so only limited data would be obtained if HHV-1/2 testing had been undertaken. Furthermore, although commercially available type specific serological tests are available for the detection of HHV-1/2 antibodies obtaining reliable data for HHV-2 is subject to a number of technical limitations (268). These include significant numbers of false-positive results in low prevalence populations, delayed seroconversion, sero-reversion (269). Any meaningful study of HHV-1/2 would need to address the aforementioned issues. Previous studies (199, 214, 216) have failed to show an association of HHV-1/2 antibodies with multiple sclerosis.

There are two variants of human herpesvirus 6 (HHV-6A and HHV-6B) and both these and human herpesvirus 7 (HHV-7) can be cultured from peripheral blood mononuclear cells and are classified in the subfamily *Betaherpesvirinae*. They are associated with the childhood disease roseola (exanthem subitem) to differing extents (mainly HHV-6B). Seizures and severe encephalitis have been linked to primary infection and reactivation in immunocompromised patients respectively and it has been proposed to have an association with multiple sclerosis (270). Antigenic cross-reactivity between HHV-6 and HHV-7 limits the usefulness of serological assays (271) so, although it

would have been interesting to investigate HHV-6 antibody levels in the WIRMS sera any data obtained would be compromised. Finally, HHV-8 is the most recently discovered of the human herpesviruses (272) and there is little reason to consider a potential association of this herpesvirus with multiple sclerosis (273).

6. Human herpesvirus infections may act as markers (see Discussion) for risk of multiple sclerosis and severity of disease. It would be interesting to see whether clinically isolated syndrome patients show similar serological characteristics.

7. There has been considerable interest in the influence of host microbiome in the generation of disease and, within this, the host virome may play a very significant role. We should endeavour to add to this body of knowledge.

6.0 REFERENCES

1. Murray TJ. The history of multiple sclerosis: the changing frame of the disease over the centuries. J Neurol Sci 2009; 277, Suppl 1: S3-S8.

2. Firth D. The case of Augustus d'Este (1794-1848): the first account of disseminated sclerosis: (section of the history of medicine). Proc R Soc Med 1941; 34: 381-384.

3. Bogousslavsky J, Walusinski O, Moulin T. Alfred Vulpian and Jean-Martin Charcot in each other's shadow? From Castor and Pollux at La Salpêtrière to neurology forever. Eur Neurol 2011; 65: 215-222.

4. Compston A. The 150th anniversary of the first depiction of the lesions of multiple sclerosis. J Neurol Neurosurg Psychiatry 1988; 51: 1249-1252.

5. Putnam TJ. Multiple sclerosis and "encephalomyelitis". Bull N Y Acad Med 1943; 19: 301-316.

6. Poser CM, Brinar VV. Diagnostic criteria for multiple sclerosis: an historical review. Clin Neurol Neurosurg 2004; 106: 147-158.

7. Poser CM, Brinar VV. Diagnostic criteria for multiple sclerosis. Clin Neurol Neurosurg 2001; 103: 1-11.

8. Poser CM, Brinar VV. Problems with diagnostic criteria for multiple sclerosis. Lancet 2001; 358: 1746-1747.

9. Sahraian MA, Eshaghi A. Role of MRI in diagnosis and treatment of multiple sclerosis. Clin Neurol Neurosurg 2010; 112: 609-615.

10. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, *et al*. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 2001; 50: 121-127.

11. Miller DH, Filippi M, Fazekas F, Frederiksen JL, Matthews PM, Montalban X, *et al*. Role of magnetic resonance imaging within diagnostic criteria for multiple sclerosis. Ann Neurol 2004; 56: 273-278.

12. Dalton CM, Brex PA, Miszkiel KA, Hickman SJ, MacManus DG, Plant GT, *et al*. Application of the new McDonald criteria to patients with clinically isolated syndromes suggestive of multiple sclerosis. Ann Neurol 2002; 52: 47-53.

13. Polman CH, Reingold SC, Edan G, Filippi M, Hartung H-P, Kappos L, *et al*. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Ann Neurol 2005; 58: 840-846.

14. Ntranos A, Lublin F. Diagnostic criteria, classification and treatment goals in multiple sclerosis: the chronicles of time and space. Curr Neurol Neurosci Rep 2016; 16: 90.

15. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, *et al*. Diagnostic criteria for multiple sclerosis: 2010 revisions to the "McDonald Criteria". Ann Neurol 2011; 69: 292-302.

16. Perry M, Swain S, Kemmis-Betty S, Cooper P, Guideline Development Group of the National Institute for Health and Clinical Excellence. BMJ 2014; 349: g5701.

17. Solomon AJ, Bourdette DN, Cross AH, Applebee A, Skidd PM, Howard DB, *et al*. The contemporary spectrum of multiple sclerosis misdiagnosis: A multicentre study. Neurology 2016; 87: 1393-1399.

18. Brownlee WJ, Hardy TA, Fazekas F, Miller DH. Diagnosis of multiple sclerosis: progress and challenges. Lancet doi: 10.1016/S0140-6736(16)30959-X. Epub 2016 Nov 23.

19. Bjartmar C, Wujek JR, Trapp BD. Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. J Neurol Sci 2003; 206: 165-171.

20. Lassmann H. Pathology and disease mechanisms in different stages of multiple sclerosis. J Neurol Sci 2013; 333: 1-4.

21. Keegan BM, Noseworthy JH. Multiple sclerosis. Annu Rev Med 2002; 53: 285-302.

22. Lassmann H. Multiple sclerosis: lessons from molecular neuropathology. Exp Neurol 2014; 262: 2-7.

23. Kingwell E, Marriott JJ, Jette N, Pringsheim T, Makhani N, Morrow SA, *et al*. Incidence and prevalence of multiple sclerosis in Europe: a systematic review. BMC Neurol 2013; 13: 128.

24. Hirst C, Ingram G, Pickersgill T, Swingler R, Compton DAS, Robertson NP. Increasing prevalence and incidence of multiple sclerosis in South East Wales. J Neurol Neurosurg Psychiatry 2009; 80: 386-391.

25. Gray OM, McDonnell GV, Hawkins SA. Factors in the rising prevalence of multiple sclerosis in the north-east of Ireland. Mult Scler 2008; 14: 880-886.

26. Mackenzie IS, Morant SV, Bloomfield GA, MacDonald TM, O'Riordan JO. Incidence and prevalence of multiple sclerosis in the UK 1990-2010: a descriptive study in the General Practice Research Database. J Neurol Neurosurg Psychiatry 2014; 85: 76-84.

27. Taylor R, Illsley R, Poskanzer DC. Multiple sclerosis in the Orkney and Shetland Islands VI: The effects of migration and social structure. J Epidemiol Community Health 1980; 34: 271-276.

28. Rothwell PM, Charlton D. High incidence and prevalence of multiple sclerosis in south east Scotland: evidence of a genetic predisposition. J Neurol Neurosurg Psychiatry 1998; 64: 730-735.

29. Mumford CJ, Fraser MB, Wood NW, Compston DAS. Multiple sclerosis in the Cambridge health district of East Anglia. J Neurol Neurosurg Psychiatry 1992; 55: 877-882.

30. Ford HL, Gerry E, Airey CM, Vail A, Johnson MH, Williams DRR. The prevalence of multiple sclerosis in the Leeds Health Authority. J Neurol Neurosurg Psychiatry 1998; 64: 605-610.

31. Browne P, Chandraratna D, Angood C, Tremlett H, Baker C, Taylor BV, *et al*. Atlas of multiple sclerosis 2013: a growing global problem with widespread inequity. Neurology 2014: 83: 1022-1024.

32. Makhani N, Morrow SA, Fisk J, Evans C, Beland SG, Kulaga S, *et al*. MS incidence and prevalence in Africa, Asia, Australia and New Zealand: a systematic review. Mult Scler Relat Disord 2014; 3: 48-60.

33. Heydarpour P, Khoshkish S, Abtahi S, Moradi-Lakeh M, Sahraian MA. Multiple sclerosis epidemiology in Middle East and North Africa: a systematic review and meta-analysis. Neuroepidemiology 2015; 44: 232-244.

34. Schiess N, Huether K, Fatafta T, Fitzgerald KC, Calabresi PA, Blair I, *et al*. How global MS prevalence is changing: a retrospective chart review in the United Arab Emirates. Mult Scler Relat Disord 2016; 9: 73-79.

35. Trojano M, Lucchese G, Graziano G, Taylor BV, Simpson S Jr, Lepore V, *et al*. Geographical variations in sex ratio trends over time in multiple sclerosis. PLoS ONE 2012; 7(10): e48078.

36. Koch-Henriksen N, Soelberg Sorensen P. The changing demographic pattern of multiple sclerosis epidemiology. Lancet Neurol 2010; 9: 520-532.

37. Etemadifar M, Sajjadi S, Nasr Z, Firoozeei TS, Abtahi SH, Akbari M, *et al*. Epidemiology of multiple sclerosis in Iran: a systematic review. Eur Neurol 2013; 70: 356-363.

38. Alonso A, Jick SS, Olek MJ, Hernán MA. Incidence of multiple sclerosis in the United Kingdom: findings from a population-based cohort. J Neurol 2007; 254: 1736-1741.

39. Zuvich RL, McCauley JL, Pericak-Vance MA, Haines JL. Genetics and pathogenesis of multiple sclerosis. Semin Immunol 2009; 21: 328-333.

40. Olsson T, Barcellos LF, Alfredsson L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. Nat Rev Neurol 2017; 13: 25-36.

41. Milo R, Kahana E. Multiple sclerosis: geoepidemiology, genetics and the environment. Autoimmun Rev 2010; 9: A387-A394.

42. Mokry LE, Ross S, Ahmad OS, Forgetta V, Smith GD, Leong A, *et al*. Vitamin D and risk of multiple sclerosis: a Mendelian randomization study. PLoS Med 2015; 12(8): e1001866.

43. Hart PH, Gorman S. Exposure to UV wavelengths in sunlight suppresses immunity. To what extent is UV-induced vitamin D_3 the mediator responsible? Clin Biochem Rev 2013; 34: 3-13.

44. Jörg S, Grohme DA, Erzler M, Binsfeld M, Haghikia A, Müller D, *et al*. Environmental factors in autoimmune diseases and their role in multiple sclerosis. Cell Mol Life Sci 2016; 73: 4611-4622.

45. Hartung HP, Aktas O, Menge T, Kieseier BC. Immune regulation of multiple sclerosis. Handb Clin Neurol 2014; 122: 3-14.

46. Criste C, Trapp B, Dutta R. Axonal loss in multiple sclerosis: causes and mechanisms. Handb Clin Neurol 2014; 122: 101-113.

47. Selmi C, Lu Q, Humble MC. Heritability versus the role of the environment in autoimmunity. J Autoimmun 2012; 39: 249-252.

48. Kurtzke JF. Epidemiologic evidence for multiple sclerosis as an infection. Clin Microbiol Rev 1993; 6: 382-427.

49. Gilden DH. Infectious causes of multiple sclerosis. Lancet Neurol 2005; 4: 195-202.

50. Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. Lancet Neurol 2004; 3: 104-110.

51. Baranzini SE, Wang J, Gibson RA, Galwey N, Naegelin Y, Barkhof F, *et al*. Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. Hum Mol Genet 2009; 18: 767-778.

52. Joseph FG, Hirst CL, Pickersgill TP, Ben-Shlomo Y, Robertson NP, Scolding NJ. CSF oligoclonal band status informs prognosis in multiple sclerosis: a case control study of 100 patients. J Neurol Neurosurg Psychiatry 2009; 80: 292-296.

53. Owens GP, Bennett JL, Gilden DH, Burgoon MP. The B cell response in multiple sclerosis. Neurol Res 2006; 28: 236-244.

54. Gale CR, Martyn CN. Migrant studies in multiple sclerosis. Prog Neurobiol 1995; 47: 425-448.

55. Joensen P. Multiple sclerosis: variation of incidence of onset over time in the Faroe Islands. Mult Scler 2011; 17: 241-244.

56. Marrie RA. Environmental risk factors in multiple sclerosis aetiology. Lancet Neurol 2004; 3: 709-718.

57. Pender MP, Burrows SR. Epstein-Barr virus and multiple sclerosis: potential opportunities for immunotherapy. Clin Transl Immunology 2014 Oct 31; 3: e27.

58. Subramaniam S, Steiner I. *Chlamydia pneumoniae*: a candidate pathogen. Lancet Neurol 2006; 5: 891-892.

59. Morandi E, Tarlinton RE, Gran B. Multiple sclerosis between genetics and infections: human endogenous retroviruses in monocytes and macrophages. Mult Scler 2017; doi 10.1177/1352458517704711. Epub Apr 1.

60. Marrack P, Kappler J, Kotzin BL. Autoimmune disease: why and where it occurs. Nat Med 2001; 7: 899-905.

61. Rioux JD, Abbas AK. Paths to understanding the genetic basis of autoimmune disease. Nature 2005; 435: 584-589.

62. Cooper GS, Bynum MLK, Somers EC. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. J Autoimmun 2009; 33: 197-207.

63. Gale EAM. The rise of childhood type 1 diabetes in the 20th Century. Diabetes 2002; 51: 3353-3361.

64. Cooper GS, Stroehla BC. The epidemiology of autoimmune diseases. Autoimmun Rev 2003; 2: 119-125.

65. Okada H, Kuhn C, Feillet H, Bach JF. The “hygiene hypothesis” for autoimmune and allergic diseases: an update. Clin Exp Immunol 2010; 160: 1-9.

66. Strachan DP. Hay fever, hygiene, and household size. BMJ 1989; 299: 1259-1260.

67. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002; 347: 911-920.

68. Chapman NM, Coppieters K, von Herrath M, Tracy S. The microbiology of human hygiene and its impact on type 1 diabetes. Islets 2012; 4: 253-261.

69. Kondrashova A, Seiskari T, Ilonen J, Knip M, Hyoty H. The “Hygiene hypothesis” and the sharp gradient in the incidence of autoimmune and allergic diseases between Russian Karelia and Finland. APMIS 2013; 121: 478-493.

70. Ahlgren C, Lycke J, Odén A, Andersen O. High risk of MS in Iranian immigrants in Gothenburg, Sweden. Mult Scler 2010; 16: 1079-1082.

71. Bach JF. Infections and autoimmune diseases. J Autoimmun 2005; 25: 74-80.

72. Versini M, Jeandel PY, Bashi T, Bizzaro G, Blank M, Shoenfeld Y. Unraveling the Hygiene Hypothesis of helminths and autoimmunity: origins, pathophysiology, and clinical applications. BMC Med 2015; 13: 81.

73. Wiria AE, Djuardi Y, Supali T, Sartono E, Yazdanbakhsh M. Helminth infection in populations undergoing epidemiological transition: a friend or foe? Semin Immunopathol 2012; 34: 889-901.

74. Sanya RE, Nkurunungi G, Biraro IA, Mpairwe H, Elliott AM. A life without worms. Trans R Soc Trop Med Hyg 2017; 111: 3-11.

75. Lynch NR, Hagel I, Perez M, Di Prisco MC, Lopez R, Alvarez N. Effect of antihelminthic treatment on the allergic reactivity of children in a tropical slum. J Allergy Clin Immunol 1993; 92: 404-411.

76. McSorley HJ, Malzels RM. Helminth infections and host immune regulation. Clin Microbiol Rev 2012; 25: 585-608.

77. Allen JE, Wynn TA. Evolution of Th2 immunity: a rapid repair response to tissue destructive pathogens. PLoS Pathog 2011; 7:e1002003.

78. Fleming JO, Cook TD. Multiple sclerosis and the hygiene hypothesis. Neurology 2006; 67: 2085-2086.

79. Cabre P. Environmental changes and epidemiology of multiple sclerosis in the French West Indies. J Neurol Sci 2009; 286: 58-61.

80. Smallwood TB, Giacomin PR, Loukas A, Mulvenna JP, Clark RJ, Miles JJ. Helminth immodulation in autoimmune disease. Front Immunol 2017; 8: 453.

81. Correale J, Farez M. Association between parasite infection and immune responses in multiple sclerosis. Ann Neurol 2007; 61: 97-108.

82. Correale J, Farez MF. The impact of parasite infections on the course of multiple sclerosis. J Neuroimmunol 2011; 233: 6-11.

83. Murray TJ. Multiple sclerosis: the history of a disease. New York: Demos; 2005.

84. Maple PA, al-Wali W. The prevention of tetanus in England and Wales. Commun Dis Public Health 2001; 4:106-113.

85. McAlpine D. An address on DISSEMINATED SCLEROSIS. Br Med J 1927; 1(3449): 269-273.

86. Schumacher GA. Multiple sclerosis. J Am Med Assoc 1950; 143:1146-1154.

87. Schumacher GA. Treatment of multiple sclerosis: a review of drug therapy. J Am Med Assoc 1950; 143: 1241-1250.

88. McAlpine D. Multiple sclerosis: a plea for a fresh outlook. Br Med J 1957; 1(5017): 475-480.

89. Miller H, Schapira K. Aetiological aspects of multiple sclerosis. Part II. Br Med J 1959; 1(5125): 811-815.

90. Roboz-Einstein E. Allergic encephalomyelitis as an experimental model for multiple sclerosis. Calif Med 1959; 91: 204-206.

91. Joint Committee of the Medical Research Council and Nuffield Foundation on clinical trials of cortisone, ACTH, and other therapeutic measures in chronic rheumatic diseases. A comparison of prednisolone with aspirin or other analgesics in the treatment of rheumatoid arthritis. Ann Rheum Dis 1959; 18: 173-188.

92. Miller H, Newell DJ, Ridley A. Multiple sclerosis. Trials of maintenance treatment with prednisolone and soluble aspirin. Lancet 1961; 1(7169): 127-129.

93. Andersson PB, Goodkin DE. Glucocorticosteroid therapy for multiple sclerosis: a critical review. J Neurol Sci 1998; 160: 16-25.

94. Lattanzi S, Cagnetti C, Danni M, Provinciali L, Silvestrini M. Oral and intravenous steroids for multiple sclerosis relapse: a systematic review and meta-analysis. J Neurol. Epub 2017 May 10. doi 10.1007/s00415-017-8505-0.

95. Ontaneda D, Thompson AJ, Fox RJ, Cohen JA. Progressive multiple sclerosis: prospects for disease therapy, repair, and restoration of function. Lancet 2017; 389(10076): 1357-1366.

96. Isaacs A, Lindemann J. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 1957; 147: 258-267.

97. Hughes RAC. Immunotherapy for multiple sclerosis. J Neurol Neurosurg Psychiatry 1994; 57: 3-6.

98. Markowitz C. Development of interferon-beta as a therapy for multiple sclerosis. Expert Opin Emerg Drugs 2004; 9: 363-374.

99. Soini E, Joutseno J, Sumelahti ML. Cost-utility of first-line disease-modifying treatments for relapsing-remitting multiple sclerosis. Clin Ther 2017; 39: 537-557.

100. Oliver BJ, Kohli E, Kasper LH. Interferon therapy in relapsing-remitting multiple sclerosis: a systematic review and meta-analysis of the comparative trials. J Neurol Sci 2011; 302: 96-105.

101. Applebee A, Panitch H. Early stage and long term treatment of multiple sclerosis with interferon-β. Biologics 2009; 3: 257-271.

102. Fernández O, Delvecchio M, Edan G, Fredrikson S, Gionvannoni G, Hartung HP, *et al*. Survey of diagnostic and treatment practices for multiple sclerosis in Europe.

103. Rostami Yazdi M, Mrowietz U. Fumaric acid esters. Clin Dermatol 2008; 26: 522-526.

104. Schilling S, Goelz S, Linker R, Luehder F, Gold R. Fumaric acid esters are effective in chronic experimental autoimmune encephalomyelitis and suppress macrophage infiltration. Clin Exp Immunol 2006; 145: 101-107.

105. Bomprezzi R. Dimethyl fumarate in the treatment of relapsing-remitting multiple sclerosis: an overview. Ther Adv Neurol Disord 2015; 8: 20-30.

106. Smoot K, Spinelli KJ, Stuchiner T, Lucas L, Chen C, Grote L, *et al*. Three-year clinical outcomes of relapsing multiple sclerosis patients treated with dimethyl fumarate in a United States community health center. Mult Scler Epub 2017 May 1, 1-9.

107. Gieselbach RJ, Muller-Hansma AH, Wijburg MT, de Bruin-Weller MS, van Oosten BW, Nieuwkamp DJ, *et al*. Progressive multifocal leukoencephalopathy in patients treated with fumaric acid esters: a review of 19 cases. J Neurol 2017; 264: 1155-1164.

108. Merrill JE, Hanak S, Pu SF, Liang J, Dang C, Iglesias-Bregna D, *et al*. Teriflunomide reduces behavioural, electrophysiological, and histopathological deficits in the Dark Agouti rat model of experimental autoimmune encephalomyelitis. J Neurol 2009; 256: 89-103.

109. Bar-Or A, Pachner A, Menguy-Vacheron F, Kaplan J, Wiendl H. Teriflunomide and its mechanism of action in multiple sclerosis. Drugs 2014; 74: 659-674.

110. Kasarello K, Cudnoch-Jędrzejewska A, Czlonkowski A, Mirowska-Guzel D. Mechanism of action of three newly registered drugs for multiple sclerosis treatment. Pharmacol Rep 2017; 69: 702-708.

111. Kieseier BC, Wiendl H. New evidence for teriflunomide in multiple sclerosis. Lancet Neurol 2014; 13: 234-235.

112. Confavreux C, O'Connor P, Comi G, Freedman MS, Miller AE, Olsson TP, *et al*. Oral teriflunomide for patients with relapsing multiple sclerosis (TOWER): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Neurol 2014; 13: 247-256.

113. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256: 495-497.

114. Beck A, Wurch T, Bailly C, Corvaia N. Strategies and challenges for the next generation of therapeutic antibodies. Nat Rev Immunol 2010; 10: 345-352.

115. Wingerchuk DM, Weinshenker BG. Disease modifying therapies for relapsing multiple sclerosis. BMJ 2016; 354: i3518.

116. Hartung HP, Aktas O, Boyko AN. Alemtuzumab: A new therapy for active relapsing-remitting multiple sclerosis. Mult Scler 2015; 21: 22-34.

117. Preiningerova JL, Vachova M. Daclizumab high-yield process in the treatment of relapsing-remitting multiple sclerosis. Ther Adv Neurol Disord 2017; 10: 67-75.

118. Davenport RJ, Munday JR. Alpha4-integrin antagonism – an effective approach for the treatment of inflammatory diseases? Drug Discov Today 2007; 12: 569-576.

119. Frampton JE. Ocrelizumab: First global approval. Drugs 2017; 77: 1035-1041.

120. Nguyen AL, Gresle M, Marshall T, Butzkueven H, Field J. Monoclonal antibodies in the treatment of multiple sclerosis: emergence of B-cell-targeted therapies. Br J Pharmacol 2017; 174: 1895-1907.

121. Summers RW, Elliott DE, Urban JF Jr, Thompson R, Weinstock JV. *Trichuris suis* therapy in Crohn's disease. Gut 2005; 54: 87-90.

122. Helmby H. Human helminth therapy to treat inflammatory disorders – where do we stand? BMC Immunol 2015; 16: 12.

123. Hasseldam H, Hansen CS, Johansen FF. Immunomodulatory effects of helminths and protozoa in multiple sclerosis and experimental autoimmune encephalomyelitis. Parasite Immunol 2013; 35: 103-108.

124. Lund ME, Greer J, Dixit A, Alvarado R, McCauley-Winter P, To J, *et al*. A parasite-derived 68-mer peptide ameliorates autoimmune disease in murine models of type 1 diabetes and multiple sclerosis. Sci Rep 2016; 6: 37789.

125. Tanasescu R, Constantinescu CS. Helminth therapy for MS. Curr Top Behav Neurosci 2015; 26: 195-220.

126. Wammes LJ, Mpairwe H, Elliott AM, Yazdanbakhsh M. Helminth therapy or elimination: epidemiological, immunological, and clinical considerations. Lancet Infect Dis 2014; 14: 1150-1162.

127. Periago MV, Bethony JM. Hookworm virulence factors: making the most of the host. Microbes Infect 2012; 14: 1451-1464.

128. Fleming JO. Helminth therapy and multiple sclerosis. Int J Parasitol 2013; 43: 259-274.

129. Gaze S, McSorley HJ, Daveson J, Jones D, Bethony JM, Oliveira LM, *et al*. Characterising the mucosal and systemic immune responses to experimental human hookworm infection. PLoS Pathog 2012; 8: e1002520.

130. Osborne LC, Monticelli LA, Nice TJ, Sutherland TE, Siracusa MC, Hepworth MR, *et al*. Coinfection. Virus-helminth coinfection reveals a microbiota-independent mechanism of immunomodulation. Science 2014; 345: 578-582.

131. Roizman B, Baines J. The diversity and unity of *Herpesviridae*. Comp Immunol Microbiol Infect Dis 1991; 14: 63-79.

132. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC *et al*. The order *Herpesvirales*. Arch Virol 2009; 154: 171-177.

133. Davison AJ. Herpesvirus systematics. Vet Microbiol 2010; 143: 52-69.

134. Davison AJ, Scott JE. The complete DNA sequence of varicella-zoster virus. J Gen Virol 1986; 67: 1759-1816.

135. Muir WB, Nichols R, Breuer J. Phylogenetic analysis of varicella-zoster virus: evidence of intercontinental spread of genotypes and recombination. J Virol 2002; 76: 1971-1979.

136. Breuer J, Grose C, Norberg P, Tipples G, Schmid DS. A proposal for a common nomenclature for viral clades that form the species varicella-zoster virus: summary of VZV nomenclature meeting 2008, Barts and the London School of Medicine and Dentistry, 24-25 July 2008. J Gen Virol 2010; 91: 821-828.

137. Harper DR, Gilbert RL, Jeffries DJ. Molecular biology of varicella-zoster virus: a review prepared for the UK advisory group on chickenpox. J Infect 1998; 36 Suppl 1: 1-10.

138. Arvin AM. Varicella-zoster virus: molecular virology and virus-host interactions. Curr Opin Microbiol 2001; 4: 442-449.

139. Vyse AJ, Gay NJ, Hesketh LM, Morgan-Capner P, Miller E. Seroprevalence of antibody to varicella zoster virus in England and

Wales in children and young adults. Epidemiol Infect 2004; 132: 1129-1134.

140. Boughton CR. Varicella-zoster in Sydney: 1. Varicella and its complications. Med J Aust 1966; 2: 392-397.

141. Leclair JM, Zaia JA, Levin MJ, Congdon RG, Goldmann DA. Airborne transmission of chickenpox in a hospital. N Engl J Med 1980; 302: 450-453.

142. Zerboni L, Sen N, Oliver SL, Arvin AM. Molecular mechanisms of varicella zoster virus pathogenesis. Nat Rev Microbiol 2014; 12: 197-210.

143. Arvin A. Aging, immunity, and the varicella-zoster virus. N Engl J Med 2005; 352: 2266-2267.

144. Schröder C, Enders D, Schink T, Riedel O. Incidence of herpes zoster amongst adults varies by severity of immunosuppression. J Infect 2017; 75: 207-215.

145. Ross RT, Nicolle LE, Cheang M. Varicella zoster virus and multiple sclerosis in a Hutterite population. J Clin Epidemiol 1995; 48: 1319-1324.

146. Rodríguez-Violante M, Ordoñez G, Bermudez JR, Sotelo J, Corona T. Association of a history of varicella virus infection with multiple sclerosis. Clin Neurol Neurosurg 2009; 111: 54-56.

147. Marrie RA, Wolfson C. Multiple sclerosis and varicella zoster virus infection: a review. Epidemiol Infect 2001; 127: 315-325.

148. Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X, *et al*. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med 2010; 362: 402-415.

149. Coles AJ, Twyman CL, Arnold DL, Cohen JA, Confavreux C, Fox EJ, *et al*. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. Lancet 2012; 380: 1829-1839.

150. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet 1964; 1(7335): 702-703.

151. Cameron KR, Stamminger T, Craxton M, Bodemer W, Honess RW, Fleckenstein B. The 160,000-M_r virion protein encoded at the right end of the herpesvirus Saimiri genome is homologous to the 140,000-M_r membrane antigen encoded at the left end of the Epstein-Barr virus genome. J Virol 1987; 61: 2063-2070.

152. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, *et al*. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 1984; 310: 207-211.

153. Dolan A, Addison C, Gatherer D, Davison AJ, McGeoch DJ. The genome of Epstein-Barr virus type 2 strain AG876. Virology 2006; 350: 164-170.

154. Sample J, Young L, Martin B, Chatman T, Kieff E, Rickinson A, Kieff E. Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. J Virol 1990; 64: 4084-4092.

155. Kwok H, Tong AHY, Lin CH, Lok S, Farrell PJ, Kwong DLW, *et al*. Genomic sequencing and comparative analysis of Epstein-Barr virus genome isolated from primary nasopharyngeal carcinoma biopsy. PLoS ONE 2012; 7: e36939.

156. Middeldorp JM, Brink AATP, van den Brule AJC, Meijer CJLM. Pathogenic roles for Epstein-Barr virus (EBV) gene products in EBV-associated proliferative disorders. Crit Rev Oncol Hematol 2003; 45: 1-36.

157. Borza CM, Hutt-Fletcher LM. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. Nat Med 2002; 8: 594-599.

158. Nemerow GR, Mold C, Schwend VK, Tollefson V, Cooper NR. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. J Virol 1987; 61: 1416-1420.

159. Nemerow GR, Wolfert R, McNaughton ME, Cooper NR. Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). J Virol 1985; 55: 347-351.

160. Cohen JI. The biology of Epstein-Barr virus: lessons learned from the virus and the host. Curr Opin Immunol 1999; 11:365-370.

161. Young LS, Dawson CW, Eliopoulos AG. The expression and function of Epstein-Barr virus encoded latent genes. Mol Pathol 2000; 53:238-247.

162. Frappier L. EBNA 1. Curr Top Microbiol Immunol 2015; 391: 3-34.

163. Lünemann JD, Jelčić I, Roberts S, Lutterotti A, Tackenberg B, Martin R, *et al*. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-γ and IL-2. J Exp Med 2008; 205: 1763-1773.

164. Geginat J, Paroni M, Pagani M, Galimberti D, de Francesco R, Scarpini E, *et al*. The enigmatic role of viruses in multiple sclerosis: molecular mimicry or disturbed immune surveillance. Trends Immunol 2017; 38: 498-512.

165. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004; 4: 757-768.

166. Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA. EBV persistence in memory B cells in vivo. Immunity 1998; 9: 395-404.

167. Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. N Engl J Med 1984; 310: 1225-1230.

168. Hislop AD, Taylor GS, Sauce D, Rickinson AB. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. Annu Rev Immunol 2007; 25: 587-617.

169. Chan KH, Tam JSL, Peiris JSM, Seto WH, Ng MH. Epstein-Barr virus (EBV) infection in infancy. J Clin Virol 2001; 21: 57-62.

170. Dowd JB, Palermo T, Brite J, McDade TW, Aiello A. Seroprevalence of Epstein-Barr virus infection in U.S. children ages 6-19, 2003-2010. PLoS One 2013; 8: e64921.

171. Morris MC, Edmunds WJ. The changing epidemiology of infectious mononucleosis. J Infect 2002; 45: 107-132.

172. Odumade OA, Hogquist KA, Balfour HH. Progress and problems in understanding and managing primary Epstein-Barr virus infections. Clin Microbiol Rev 2011; 24: 193-209.

173. Kimura H, Morishima T, Kanegane H, Ohga S, Hoshino Y, Maeda A, *et al*. Prognostic factors for chronic active Epstein-Barr virus infection. J Infect Dis 2003; 187: 527-533.

174. Shah KM, Young LS. Epstein-Barr virus and carcinogenesis: beyond Burkitt's lymphoma. Clin Microbiol Infect 2009; 15: 982-988.

175. Levitsky V, Masucci MG. Manipulation of immune responses by Epstein-Barr virus. Virus Res 2002; 88: 71-86.

176. Rezaei N, Mahmoudi E, Aghamohammadi A, Das R, Nichols KE. X-linked lymphoproliferative syndrome: a genetic condition typified by the triad of infection, immunodeficiency and lymphoma. Br J Haematol 2011; 152: 13-30.

177. Loren AW, Porter DL, Stadtmauer EA, Tsai DE. Post-transplant lymphoproliferative disorder: a review. Bone Marrow Transplant 2003; 31: 145-155.

178. Juvonen E, Aalto SM, Tarkkanen J, Volin L, Mattila PS, Knuutila S, *et al*. High incidence of PTLD after non-T-cell-depleted allogeneic haematopoietic stem cell transplantation as a consequence of intensive immunosuppressive treatment. Bone Marrow Transplant 2003; 32: 97-102.

179. Münz C, Moormann A. Immune escape by Epstein-Barr virus associated malignancies. Semin Cancer Biol 2008; 381-387.

180. Toussirot E, Roudier J. Epstein-Barr virus in autoimmune diseases. Best Pract Res Clin Rheumatol 2008; 22: 883-896.

181. Belbasis L, Bellou V, Evangelou E, Ioannidis JPA, Tzoulaki I. Environmental risk factors and multiple sclerosis: an umbrella review of systematic reviews and meta-analyses. Lancet Neurol 2015; 14: 263-273.

182. Levin LI, Munger KL, O'Reilly EJ, Ascherio A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. Ann Neurol 2010; 67: 824-830.

183. Fourcade G, Germi R, Guerber F, Lupo J, Baccard M, Seigneurin A, *et al*. Evolution of EBV seroprevalence and primary infection age in a French hospital and a city laboratory network, 2000-2016. PLoS One 2017; 12: e0175574.

184. Pakpoor J, Disanto G, Gerber JE, Dobson R, Meier UC, Giovannoni G, *et al*. The risk of developing multiple sclerosis in individuals seronegative for Epstein-Barr virus: a meta-analysis. Mult Scler 2013; 19: 162-166.

185. Handel AE, Williamson AJ, Disanto G, Handunnetthi L, Giovannoni G, Ramagopalan SV. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. PLoS One 2010; 5: e12496.

186. Sundström P, Wadell JP, Hallmans G, Svenningsson A, Nyström L, Dilner J, *et al*. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology 2004; 62: 2277-2282.

187. Lindsey JW, Hatfield LM, Vu T. Epstein-Barr virus neutralizing and early antigen antibodies in multiple sclerosis. Eur J Neurol 2010; 17: 1263-1269.

188. Larsen PD, Bloomer LC, Bray PF. Epstein-Barr nuclear antigen and viral capsid antigen antibody titers in multiple sclerosis. Neurology 1985; 35: 435-438.

189. Sumaya CV, Myers LW, Ellison GW, Ench Y. Increased prevalence and titer of Epstein-Barr virus antibodies in patients with multiple sclerosis. Ann Neurol 1985; 17: 371-377.

190. Farrell RA, Antony D, Wall GR, Clark DA, Fisniku L, Swanton J, *et al*. Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. Neurology 2009; 73: 32-38.

191. Kvistad S, Myhr KM, Holmøy T, Bakke S, Beiske AG, Bjerve KS, *et al*. Antibodies to Epstein-Barr virus and MRI disease activity in multiple sclerosis. Mult Scler 2014; 20: 1833-1840.

192. Munger KL, Fitzgerald KC, Freedman MS, Hartung HP, Miller DH, Montalbán X, *et al*. No association of multiple sclerosis activity and progression with EBV or tobacco use in BENEFIT. Neurology 2015; 85: 1694-1701.

193. Gieβ RM, Pfuhl C, Behrens JR, Rasche L, Freitag E, Khalighy N, *et al*. Epstein-Barr virus antibodies in serum and DNA load in saliva are not associated with radiological or clinical disease activity in patients with early multiple sclerosis. PLoS ONE 2017; 12: e0175279.

194. Wandinger K, Jabs W, Siekhaus A, Bubel S, Trillenberg P, Wagner H, *et al*. Association between clinical disease activity and Epstein-Barr virus reactivation in MS. Neurology 2000;55: 178-184.

195.Buljevac D, van Doornum GJJ, Flach HZ, Groen J, Osterhaus ADME, Hop W, *et al*. Epstein-Barr virus and disease activity in multiple sclerosis. J Neurol Neurosurg Psychiatry 2005; 76: 1377-1381.

196. Latham LB, Lee MJ, Lincoln JA, Ji N, Forsthuber TG, Lindsey JW. Antivirus immune activity in multiple sclerosis correlates with MRI activity. Acta Neurol Scand 2016; 133: 17-24.

197. Simpson S Jr, Taylor B, Burrows J, Dwyer DE, Taylor J, Ponsonby AL, *et al*. EBV & HHV6 reactivation is infrequent and not associated with MS clinical course. Acta Neurol Scand 2014; 130: 328-337.

198. Wunsch M, Hohmann C, Milles B, Rostermund C, Lehmann PV, Schroeter M, *et al*. The correlation between the virus- and brain antigen-specific B cell response in the blood of patients with multiple sclerosis. Viruses 2016; 8: 105.

199. Myhr KM, Riise T, Barrett-Connor E, Myrmel H, Vedeler C, Grønning M, *et al*. Altered antibody pattern to Epstein-Barr virus but not to other herpesviruses in multiple sclerosis: a population based case-control study from western Norway. J Neurol Neurosurg Psychiatry 1998; 64:539-542.

200. Dudgeon JA. Cytomegalovirus infection. Arch Dis Child 1971; 46: 581-583.

201. Dolan A, Cunningham C, Hector RD, Aycan F, Hassan-Walker LL, Lee L, *et al*. Genetic content of wild-type human cytomegalovirus. J Gen Virol 2004; 85: 1301-1312.

202. Huang ES, Kilpatrick BA, Huang YT, Pagano JS. Detection of human cytomegalovirus and analysis of strain variation. Yale J Biol Med 1976; 49: 29-43.

203. Dal Monte P, Pignatelli S, Rossini G, Landini MP. Genomic variants among human cytomegalovirus (HCMV) clinical isolates: the glycoprotein N (gN) paradigm. Hum Immunol 2004; 65: 387-394.

204. Gerna G, Baldanti F, Revello MG. Pathogenesis of human cytomegalovirus infection and cellular targets. Hum Immunol 2004; 65: 381-386.

205. Söderberg-Nauclér C, Fish KN, Nelson JA. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell 1997; 91: 119-126.

206. Goodrum F. Human cytomegalovirus latency: approaching the Gordian Knot. Annu Rev Virol 2016; 3: 333-357.

207. Staras SAS, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. Clin Infect Dis 2006; 43: 1143-1151.

208. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol 2010; 20: 202-213.

209. Vyse AJ, Hesketh LM, Pebody RG. The burden of infection with cytomegalovirus in England and Wales: how many women are infected in pregnancy? Epidemiol Infect 2009; 137: 526-533.

210. Ross SA, Boppana SB. Congenital cytomegalovirus infection: outcome and diagnosis. Semin Pediatr Infect Dis 2005; 16: 44-49.

211. Grosse SD, Ross DS, Dollard SC. Congenital cytomegalovirus (CMV) infection as a cause of permanent bilateral hearing loss: a quantitative assessment. J Clin Virol 2008; 41: 57-62.

212. Salzberger B, Hartmann P, Hanses F, Uyanik B, Cornely OA, Wöhrmann A, *et al*. Incidence and prognosis of CMV disease in HIV-infected patients before and after introduction of combination antiretroviral therapy. Infection 2005; 33: 345-349.

213. Husain S, Pietrangeli CE, Zeevi A. Delayed onset CMV disease in solid organ transplant recipients. Transpl Immunol 2009; 21: 1-9.

214. Banwell B, Krupp L, Kennedy J, Tellier R, Tenembaum S, Ness J, *et al*. Clinical features and viral serologies in children with multiple sclerosis: a multinational observational study. Lancet Neurol 2007; 6: 773-781.

215. Najafi S, Ghane M, Poortahmasebi V, Jazayeri SM, Yousefzadeh-Chabok S. Prevalence of cytomegalovirus in patients with multiple sclerosis: a case-control study in northern Iran. Jundishapur J Microbiol 2016; 9: e36582.

216. Waubant E, Mowry EM, Krupp L, Chitnis T, Yeh EA, Kuntz N, *et al*. Common viruses associated with lower pediatric multiple sclerosis risk. Neurology 2011; 76: 1989-1995.

217. Sundqvist E, Bergström T, Daialhosein H, Nyström M, Sundström P, Hillert J, *et al*. Cytomegalovirus seropositivity is negatively associated with multiple sclerosis. Mult Scler 2014; 20: 165-173.

218. Zivadinov R, Chin J, Horakova D, Bergsland N, Weinstock-Guttman B, Tamaño-Blanco M, *et al*. Humoral responses to herpesviruses are associated with neurodegeneration after a demyelinating event: results from the multi-center SET study. J. Neuroimmunol 2014; 273: 58-64.

219. Vanheusden M, Broux B, Welten SPM, Peeters LM, Panagioti E, Van Wijmeersch B, *et al*. Cytomegalovirus infection exacerbates autoimmune mediated neuroinflammation. Sci Rep 2017; 7: 663.

220. Zaguia F, Saikali P, Ludwin S, Newcombe J, Beauseigle D, McCrea E, *et al*. Cytotoxic NKG2C^{+} CD4 T cells target oligodendrocytes in multiple sclerosis. J Immunol 2013; 190: 2510-2518.

221. Thewissen M, Somers V, Hellings N, Fraussen J, Damoiseaux J, Stinissen P. CD4^{+}CD28^{null} T cells in autoimmune disease: pathogenic features and decreased susceptibility to immunoregulation. J Immunol 2007; 179: 6514-6523.

222. Milovanovic J, Popovic B, Milovanovic M, Kvestak D, Arsenijevic A, Stojanovic B, *et al*. Murine cytomegalovirus infection induces susceptibility to EAE in resistant BALB/c mice.

223. Vanheusden M, Stinissen P, 't Hart BA, Hellings N. Cytomegalovirus: a culprit or protector in multiple sclerosis. Trends Mol Med 2015; 21: 16-23.

224. Comi G, Radaelli M, Soelberg Sørensen P. Evolving concepts in the treatment of relapsing multiple sclerosis. Lancet 2017; 389: 1347-1356.

225. Reese TA, Wakeman BS, Choi HS, Hufford MM, Huang SC, Zhang X. Helminth infection reactivates latent γ-herpesvirus via cytokine competition at a viral promoter. Science 2014; 345: 573-577.

226. Arvin AM, Wolinsky JS, Kappos L, Morris MI, Reder AT, Tornatore C, *et al*. Varicella-Zoster virus infections in patients treated with

fingolimod: risk assessment and consensus recommendations for management. JAMA Neurol 2015; 72: 31-39.

227. Uitdehaag BM,Barkhof F, Coyle PK, Gardner JD, Jeffery DR, Mikol DD. The changing face of multiple sclerosis clinical trial populations. Curr Med Res Opin 2011; 27: 1529-1537.

228. Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on clinical trials of new agents in multiple sclerosis. Neurology 1996; 46: 907-911.

229. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Soelberg-Sørensen P, Thompson AJ, *et al*. Defining the clinical course of multiple sclerosis. The 2013 revisions. Neurology 2014; 83: 278-286.

230. Brück W. The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. J Neurol 2005; 252 (Suppl 5): v3-v9.

231. Dargahi N, Katsara M, Tselios T, Androutsou M-E, de Courten M, Matsoukas J, *et al*. Multiple sclerosis: immunopathology and treatment update. Brain Sci 2017; 7: pii: E78.

232. Stinissen P, Raus J, Zhang J. Autoimmune pathogenesis of multiple sclerosis: role of autoreactive T lymphocytes and new immunotherapeutic strategies. Crit Rev Immunol 1997; 17: 33-75.

233. Pettinelli CB, McFarlin DE. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after *in vitro* activation of lymph node cells by myelin basic protein: requirement for Lyt 1 + 2- T lymphocytes. J Immunol 1981; 127: 1420-1423.

234. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; 136: 2348-2357.

235. Steinman L. A brief history of T_H17, the first major revision in the T_H1/T_H2 hypothesis of T cell-mediated tissue damage. Nat Med 2007; 13: 139-145.

236. Awasthi A, Murugaiyan G, Kuchroo VK. Interplay between effector Th_{17} and regulatory T cells. J Clin Immunol 2008; 28: 660-670.

237. Qu N, Xu M, Mizoguchi I, Furusawa J-I, Kaneko K, Watanabe K, *et al*. Pivotal roles of T-helper 17-related cytokines, IL-17, IL-22, and IL-23, in inflammatory diseases. Clin Dev Immunol 2013; 2013: 968549.

238. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, *et al*. Fate mapping of interleukin 17-producing T cells in inflammatory responses. Nat Immunol 2011; 12: 255-263.

239. Benoist C, Mathis D. Treg cells, life history, and diversity. Cold Spring Harb Perspect Biol 2012; 4:a007021.

240. Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. Immunol Rev 2008; 223: 371-390.

241. Haas J, Hug A, Viehöver A, Fritzsching B, Falk CS, Filser A, *et al*. Reduced suppressive effect of $CD4^{+}CD25^{high}$ regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. Eur J Immunol 2005; 35: 3343-3352.

242. de Andrés C, Aristimuño C, de las Heras V, Martínez-Ginés ML, Bartolomé M, Arroyo R, *et al*. Interferon beta-1a therapy enhances $CD4^{+}$ regulatory T-cell function: An *ex vivo* and *in vitro* longitudinal study in relapsing-remitting multiple sclerosis. J Neuroimmunol 2007; 182: 204-211.

243. Comabella M, Montalban X. Body fluid biomarkers in multiple sclerosis. Lancet Neurol 2014; 13: 113-126.

244. Petzold A. Intrathecal oligoclonal IgG synthesis in multiple sclerosis. J Neuroimmunol 2013; 262: 1-10.

245. Krumbholz M, Meinl E. B cells in MS and NMO: pathogenesis and therapy. Semin Immunopathol 2014; 36: 339-350.

246. Krumbholz M, Derfuss T, Hohlfeld R, Meinl E. B cells and antibodies in multiple sclerosis pathogenesis and therapy. Nat Rev Neurol 2012; 8: 613-623.

247. Staun-Ram E, Miller A. Effector and regulatory B cells in multiple sclerosis. Clin Immunol 2017; http://dx.doi.org/10.1016/j.clim.2017.04.014.

248. Lisak RP, Nedelkoska L, Benjamins JA, Schalk D, Bealmear B, Touil H, *et al*. B cells from patients with multiple sclerosis induce cell death *via* apoptosis in neurons *in vitro*. J. Neuroimmunol 2017; 309: 88-99.

249. Cheng Y, Sun L, Xie Z, Fan X, Cao Q, Han J, *et al*. Diversity of immune cell types in multiple sclerosis and its animal model: pathological and therapeutic implications. J Neurosci Res 2017; 95: 1973-1983.

250. Munch M, Riisom K, Christensen T, Møller-Larsen A, Haahr S. The significance of Epstein-Barr virus seropositivity in multiple sclerosis patients? Acta Neurol Scand 1998; 97: 171-174.

251. de Paschale M, Clerici P. Serological diagnosis of Epstein-Barr virus infection: problems and solutions. World J Virol 2012; 1: 31-43.

252. Obel N, Høier-Madsen M, Kangro H. Serological and clinical findings in patients with serological evidence of reactivated Epstein-Barr virus infection. APMIS 1996; 104: 424-428.

253. Pottgiesser T, Schumacher YO, Wolfarth B, Schmidt-Trucksäss A, Bauer G. Longitudinal observation of Epstein-Barr virus antibodies in athletes during a competitive season. J Med Virol 2012; 84: 1415-1422.

254. Pembrey L, Raynor P, Griffiths P, Chaytor S, Wright J, Hall AJ. Seroprevalence of cytomegalovirus, Epstein Barr virus and varicella zoster virus among pregnant women in Bradford: a cohort study. PLoS One 2013; 8: e81881.

255. Komdewal MJ, Mollema L, Tcherniaeva I, van der Klis F, Kroes AC, Oudesluys-Murphy AM, *et al*. Cytomegalovirus infection in the Netherlands: seroprevalence, risk factors and implications. J Clin Virol 2015; 63: 53-58.

256. Horakova D, Zivadinov R, Weinstock-Guttman B, Havrdova E, Qu J, Tamaño-Blanco M, *et al*. Environmental factors associated with disease progression after the first demyelinating event: results from the multi-center SET study. PLoS One 2013; 8: e53996.

257. Zivadinov R, Nasuelli D, Tommasi MA, Serafin M, Bratina A, Ukmar M, *et al*. Positivity of cytomegalovirus antibodies predicts a better clinical and radiological outcome in multiple sclerosis patients. Neurol Res 2006; 28: 262-269.

258. Vanheusden M, Stinissen P, ‘t Hart B, Hellings N. Cytomegalovirus: a culprit or protector in multiple sclerosis? Trends Mol Med 2015; 21: 16-23.

259. Ross RT. The varicella-zoster virus and multiple sclerosis. J Clin Epidemiol 1998; 51: 533-535.

260. Manouchehrinia A, Tanasescu R, Kareem H, Jerca OP, Jabeen F, Shafei R, *et al*. Prevalence of a history of prior varicella/herpes zoster infection in multiple sclerosis. J Neurovirol 2017 (11 September); doi 10.1007/s13365-017-0569-1.

261. Quinlivan ML, Ayres KL, Kelly PJ, Parker SP, Scott FT, Johnson RW, *et al*. Persistence of varicella-zoster virus viraemia in patients with herpes zoster. J Clin Virol 2011; 50: 130-135.

262. Arvin AM, Koropchak CM. Immunoglobulins M and G to varicella-zoster virus measured by solid-phase radioimmunoassay: antibody responses to varicella and herpes zoster infections. J Clin Microbiol 1980; 12: 367-374.

263. Leung J, Harpaz R, Baughman AL, Heath K, Loparev V, Vázquez M, *et al*. Evaluation of laboratory methods for diagnosis of varicella. Clin Infect Dis 2010; 51: 23-32.

264. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds). *Virus Taxonomy. Classification and Nomenclature of Viruses. Ninth Report of the International Committee on Taxonomy of Viruses*. International Committee on Taxonomy of Viruses, 2012.

265. Kinchington PR, St Leger AJ, Guedon J-M, Hendricks RL. Herpes simplex virus and varicella zoster virus, the house guests who never leave. Herpesviridae 2012; 3:5.

266. Kleinschmidt-DeMasters BK, Gilden DH. The expanding spectrum of herpesvirus infections of the nervous system. Brain Pathol 2001; 440-451.

267. Steiner I, Budka H, Chaudhuri A, Koskiniemi M, Sainio K, Salonen O, *et al*. Viral meningoencephalitis: a review of diagnostic methods and guidelines for management. Eur J Neurol 2010; 17: 999-e57.

268. Ashley RL. Sorting out the new HSV type specific antibody tests. Sex Transm Infect 2001; 77: 232-237.

269. Scoular A. Using the evidence base on genital herpes: optimising the use of diagnostic tests and information provision. Sex Transm Infect 2002; ;78: 160-165.

270. De Bolle L, Naesens L, De Clercq E. Update on human herpesviruses 6 biology, clinical features, and therapy. Clin Microbiol Rev 2005; 18: 217-245.

271. Ward KN, Turner DJ, Couto Parada X, Thiruchelvam AD. Use of immunoglobulin G antibody avidity for differentiation of primary human herpesvirus 6 and 7 infections. J Clin Microbiol 2001; 39: 959-963.

272. Levy JA. Three new human herpesviruses (HHV6, HHV 7, and HHV8). Lancet 1997; 349: 558-563.

273. Gilden DH, Mahalingam R, Cohrs RJ, Tyler KL. Herpesvirus infections of the nervous system. Nat Clin Pract Neurol 2007; 3: 82-94.

7.0 APPENDICES

7.1 Qualitative EBV antibody levels in helminth treated patients

Sample/visit	OD405/650 EBV EA IgG	interpn	OD 405/650 EBV VCA IgM	interpn	OD405/650 EBV VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
Patient no 3								
WD003 v1	0.04	Neg	0.07	Neg	1.6	Pos	1.6	Pos
WD003 v8	0.04	Neg	0.08	Neg	1.6	Pos	1.6	Pos
WD003 v14	0.04	Neg	0.07	Neg	1.5	Pos	1.6	Pos
WD003 v15	0.04	Neg	0.07	Neg	1.6	Pos	1.6	Pos
Patient no 6								
SN 006 v1	0.43	Pos	0.1	Neg	1.9	Pos	1.6	Pos
SN006 v12	0.42	Pos	0.1	Neg	1.9	Pos	1.6	Pos
SN006 v15	0.43	Pos	0.11	Neg	2	Pos	1.7	Pos
Patient no 12								
JH014 v1	0.33	Pos	0.04	neg	1.93	pos	1.93	pos
JH014 v8	0.26	Pos	0.04	neg	1.93	pos	1.94	pos
JH014 v10	0.3	Pos	0.04	neg	2	pos	2	pos
JH014 v12	0.36	Pos	0.05	neg	2	pos	2	pos
JH014 v14	0.37	Pos	0.06	neg	2	pos	2	pos
JH014 v15	0.34	Pos	0.05	neg	1.99	pos	2	pos
Patient no 18								
DC018 v1	0.19	neg	0.04	neg	1.41	pos	2	pos
DC018 v6	0.16	neg	0.04	neg	1.27	pos	2	pos
DC018 v12	0.15	neg	0.04	neg	1.26	pos	2	pos
DC018 v15	0.14	neg	0.03	neg	1.31	pos	1.9	pos
Patient no 22								
RY022 v1	0.26	equiv	0.12	neg	1.45	pos	1.32	pos
RY022 v6	0.22	equiv	0.13	neg	1.4	pos	0.91	pos
RY022 v12	0.14	neg	0.09	neg	1.38	pos	0.83	pos
RY022 v15	0.2	neg	0.1	neg	1.46	pos	0.9	pos
Patient no 25								
TC025 scr	0.53	pos	0.1	neg	1.56	pos	1.3	pos
TC025 v6	0.5	pos	0.11	neg	1.48	pos	1.27	pos
TC025 v8	0.53	pos	0.12	neg	1.59	pos	1.35	pos
TC025 v10	0.51	pos	0.13	neg	1.64	pos	1.36	pos

Sample/visit	OD405/650 EBV EA IgG	interpn	OD 405/650 EBV VCA IgM	interpn	OD405/650 EBV VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
TC025 v12	0.48	pos	0.11	neg	1.64	pos	1.19	pos
TC025 v13	0.49	pos	0.11	neg	1.61	pos	1.25	pos
TC025 v15	0.46	pos	0.1	neg	1.51	pos	1.23	pos
Patient no 27								
KB028 v1	0.12	neg	0.45	equiv	1.4	pos	1.74	pos
KB028 v6	0.13	neg	0.49	equiv	1.44	pos	1.87	pos
KB028 v12	0.08	neg	0.44	equiv	1.25	pos	1.76	pos
KB028 v15	0.08	neg	0.46	equiv	1.32	pos	1.62	pos
Patient no 29								
RJ029 v1	0.1	neg	0.14	neg	1.55	pos	1.4	pos
RJ029 v6	0.09	neg	0.18	neg	1.49	pos	1.39	pos
RJ029 v12	0.09	neg	0.45	equiv	1.62	pos	1.43	pos
RJ029 v15	0.1	neg	0.15	neg	1.58	pos	1.36	pos
Patient no 33								
EW027 v1	0.08	neg	0.51	equiv	1.23	pos	1.74	pos
EW027 v6	0.07	neg	0.46	equiv	1.12	pos	1.72	pos
EW027 v12	0.08	neg	0.49	equiv	1.23	pos	1.75	pos
EW027 v15	0.08	neg	0.52	equiv	1.26	pos	1.77	pos
Patient no 36								
HW036 v1	0.3	equiv	1.37	pos	0.85	pos	2	pos
HW036 v6	0.27	equiv	1.79	pos	0.77	pos	2	pos
HW036 v12	0.32	pos	1.28	pos	0.99	pos	2.2	pos
HW036 v15	0.32	pos	1.54	pos	0.98	pos	2.2	pos
Patient no 37								
SD037 v1	0.11	neg	0.05	neg	1.41	pos	1.5	pos
SD037 v6	0.11	neg	0.05	neg	1.46	pos	1.5	pos
SD037 v12	0.11	neg	0.05	neg	1.38	pos	1.4	pos
SD037 v15	0.11	neg	0.04	neg	1.42	pos	1.4	pos
Patient no 39								
RB039 v1	0.95	Pos	0.093	Neg	1.45	Pos	1.36	Pos
RB039 v6	0.85	Pos	0.09	Neg	1.42	Pos	1.32	Pos
RB039 v12	0.89	Pos	0.14	Neg	1.44	Pos	1.33	Pos
RB039 v15	0.88	Pos	0.45	equiv	1.43	Pos	1.38	Pos
Patient no 41								
JR041 v1	0.18	neg	0.09	neg	1.55	pos	2	pos
JR041 v6	0.17	neg	0.08	neg	1.53	pos	2	pos

Sample/visit	OD405/650 EBV EA IgG	interpn	OD 405/650 EBV VCA IgM	interpn	OD405/650 EBV VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
JR041 v12	0.16	neg	0.08	neg	1.51	pos	1.9	pos
JR041 v15	0.17	neg	0.08	neg	1.58	pos	2	pos
Patient no 43								
LS043 v1	0.18	Neg	0.1	Neg	1	Pos	1.66	Pos
LS043 v6	0.18	Neg	0.11	Neg	1	Pos	1.57	Pos
LS043 v12	0.19	Neg	0.1	Neg	1	Pos	1.6	Pos
LS043 v15	0.19	Neg	0.1	Neg	1	Pos	1.66	Pos
Patient no 46								
AW045 v1	0.18	Equiv	0.04	Neg	1.3	Pos	1.7	Pos
AW045 v6	0.23	Equiv	0.05	Neg	1.3	Pos	1.7	Pos
AW045 v12	0.21	Equiv	0.04	Neg	1.3	Pos	1.7	Pos
AW045 v15	0.21	Equiv	0.04	Neg	1.4	Pos	1.7	Pos
Patient no 47								
SM046 v1	0.1	Neg	0.03	Neg	1.74	Pos	1.8	Pos
SM046 v6	0.1	Neg	0.03	Neg	1.77	Pos	1.81	Pos
SM046 v12	0.11	Neg	0.03	Neg	1.78	Pos	1.85	Pos
SM046 v15	0.1	Neg	0.02	Neg	1.68	Pos	1.75	Pos
Patient no 51								
LB051 v1	0.3	Pos	0.4	Equiv	1.9	Pos	1.8	Pos
LB051 v6	0.33	Pos	0.38	Equiv	1.9	Pos	1.8	Pos
LB051 v12	0.3	Pos	0.37	Equiv	1.8	Pos	1.7	Pos
LB051 v15	0.32	Pos	0.36	Equiv	1.8	Pos	1.7	Pos
Patient no 53								
SWC053 v1	0.07	Neg	0.07	Neg	1.5	Pos	1.7	Pos
SWC053 v6	0.07	Neg	0.08	Neg	1.5	Pos	1.6	Pos
SWC053 v12	0.07	Neg	0.07	Neg	1.5	Pos	1.7	Pos
SWC053 v15	0.07	Neg	0.08	Neg	1.5	Pos	1.6	Pos
Patient no 55								
AS055 v1	0.48	Pos	0.02	neg	1.96	pos	1.85	pos
AS055 v5	0.5	Pos	0.01	neg	2	pos	1.94	pos
AS055 v6	0.45	Pos	0.01	neg	2.1	pos	1.91	pos
AS055 v8	0.42	Pos	0.01	neg	2	pos	1.82	pos
AS055 v10	0.398	Pos	0.01	neg	1.95	pos	1.77	pos
AS055 v12	0.392	Pos	0.01	neg	1.96	pos	1.8	pos
AS055 v13	0.33	Pos	0.01	neg	1.96	pos	1.77	pos
AS055 v15	0.378	Pos	0.01	neg	1.93	pos	1.78	pos

Sample/visit	OD405/650 EBV EA IgG	interpn	OD 405/650 EBV VCA IgM	interpn	OD405/650 EBV VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
Patient no 57								
KB057 v1	0.11	Neg	0.2	Neg	1.4	Pos	1.7	Pos
KB057 v6	0.11	Neg	0.19	Neg	1.4	Pos	1.8	Pos
KB057 v12	0.09	Neg	0.19	Neg	1.4	Pos	1.7	Pos
KB057 v15	0.1	Neg	0.17	Neg	1.4	Pos	1.8	Pos
Patient no 58								
EL059 v1	0.13	Neg	0.14	Neg	1.7	Pos	1.6	Pos
EL059 v6	0.14	Neg	0.13	Neg	1.8	Pos	1.7	Pos
EL059 v12	0.14	Neg	0.13	Neg	1.8	Pos	1.7	Pos
EL059 v15	0.13	Neg	0.12	Neg	1.7	Pos	1.7	Pos
Patient no 64								
TR065 v1	0.12	neg	0.2	neg	2	pos	1.97	pos
TR065 v5	0.11	neg	0.2	neg	2	pos	2	pos
TR065 v6	0.12	neg	0.19	neg	2.1	pos	1.98	pos
TR065 v7	0.11	neg	0.2	neg	2	pos	1.92	pos
TR065 v8	0.11	neg	0.2	neg	2	pos	1.84	pos
TR065 v10	0.11	neg	0.17	neg	2	pos	1.88	pos
TR065 v12	0.13	neg	0.18	neg	2	pos	1.86	pos
TR065 v13	0.14	neg	0.18	neg	2	pos	1.87	pos
TR065 v15	0.12	neg	0.17	neg	2	pos	1.88	pos
Patient no 65								
MF066 v1	0.06	Neg	0.03	Neg	1.7	Pos	1.7	Pos
MF066 v6	0.06	Neg	0.04	Neg	1.8	Pos	1.7	Pos
MF066 v12	0.06	Neg	0.04	Neg	1.8	Pos	1.7	Pos
MF066 v15	0.06	Neg	0.03	Neg	1.7	Pos	1.7	Pos
Patient no 66								
KW067 v1	0.2	Equiv	0.2	Neg	1.2	Pos	1.6	Pos
KW067 v6	0.2	Equiv	0.17	Neg	1.1	Pos	1.6	Pos
KW067 v12	0.17	Neg	0.16	Neg	1.2	Pos	1.5	Pos
KW067 v15	0.22	Equiv	0.17	Neg	1.2	Pos	1.6	Pos
Patient no 68								
AD068 v1	0.1	Neg	0.04	Neg	1.9	Pos	1.7	Pos
AD068 v6	0.11	Neg	0.05	Neg	2	Pos	1.8	Pos
AD068 v12	0.1	Neg	0.06	Neg	1.9	Pos	1.9	Pos
AD068 v15	0.09	Neg	0.05	Neg	1.9	Pos	1.8	Pos
Patient no 70								

Sample/visit	OD405/650 EBV EA IgG	interpn	OD 405/650 EBV VCA IgM	interpn	OD405/650 EBV VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
PC070 v1	0.44	Pos	0.01	Neg	1.9	Pos	1.4	Pos
PC070 v6	0.42	Pos	0.01	Neg	1.9	Pos	1.4	Pos
PC070 v12	0.43	Pos	0.01	Neg	1.9	Pos	1.4	Pos
PC070 v15	0.6	Pos	0.01	Neg	1.9	Pos	1.5	Pos

7.2 Qualitative EBV antibody levels in placebo treated patients

Sample	OD405/650 EBV EA IgG	interpn	OD 405/650 VCA IgM	interpn	OD405/650 VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
Patient no 7								
CH007 v1	0.13	Neg	0.12	Neg	1.3	Pos	1.9	Pos
CH007 v12	0.14	Neg	0.13	Neg	1.3	Pos	1.9	Pos
CH007 v15	0.14	Neg	0.11	Neg	1.3	Pos	1.9	Pos
Patient no 9								
HC 010 v1	0.28	Pos	0.06	Neg	1.8	Pos	1.7	Pos
HC 010 v12	0.27	Pos	0.06	Neg	1.8	Pos	1.7	Pos
HC010 v15	0.27	Pos	0.06	Neg	1.8	Pos	1.6	Pos
Patient no 11								
SB011 v1	0.63	Pos	0.04	Neg	0.88	Pos	1.3	Pos
SB011 v8	0.65	Pos	0.05	Neg	0.87	Pos	1.5	Pos
SB011 v12	0.62	Pos	0.04	Neg	0.84	Pos	1.3	Pos
SB011 v15	0.63	Pos	0.04	Neg	0.87	Pos	1.3	Pos
Patient no 20								
EL020 v1	0.09	neg	0.3	neg	1.29	pos	2	pos
EL020 v6	0.1	neg	0.28	neg	1.27	pos	2	pos
EL020 v12	0.09	neg	0.3	neg	1.21	pos	1.86	pos
EL020 v15	0.09	neg	0.28	neg	1.13	pos	1.84	pos
Patient no 28								
CD031 v1	0.31	equiv	0.05	neg	1.7	pos	1.92	pos
CD031 v6	0.31	equiv	0.05	neg	1.72	pos	1.92	pos
CD031 v12	0.29	equiv	0.05	neg	1.65	pos	1.9	pos
CD031 v15	0.28	equiv	0.05	neg	1.67	pos	1.95	pos
Patient no 31								
JW032 v1	0.09	Neg	0.15	Neg	1.45	Pos	1.98	Pos
JW032 v6	0.09	Neg	0.16	Neg	1.5	Pos	1.95	Pos
JW032 v12	0.09	Neg	0.14	Neg	1.48	Pos	1.91	Pos
JW032 v15	0.07	Neg	0.14	Neg	1.37	Pos	1.95	Pos
Patient no 34								
HT034 scr	0.13	neg	0.07	neg	1.64	pos	2.08	pos
HT034 v6	0.12	neg	0.08	neg	1.73	pos	2.04	pos
HT034 v8	0.11	neg	0.07	neg	1.71	pos	2.13	pos
HT034 v10	0.1	neg	0.06	neg	1.72	pos	2	pos
HT034 v13	0.09	neg	0.06	neg	1.66	pos	1.97	pos

Sample	OD405/650 EBV EA IgG	interpn	OD 405/650 VCA IgM	interpn	OD405/650 VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
HT034 v15	0.1	neg	0.06	neg	1.64	pos	1.97	pos
Patient no 35								
MJ035 scr	0.22	equiv	0.26	neg	1.32	pos	1.53	pos
MJ035 v2	0.26	equiv	0.06	neg	1.38	pos	1.53	pos
MJ035 v6	0.26	equiv	0.04	neg	1.34	pos	1.59	pos
MJ035 v8	0.27	equiv	0.04	neg	1.36	pos	1.57	pos
MJ035 v10	0.25	equiv	0.04	neg	1.3	pos	1.55	pos
MJ035 v13	0.1	equiv	0.04	neg	1.39	pos	1.59	pos
MJ035 v15	0.25	equiv	0.03	neg	1.45	pos	1.65	pos
Patient no 38								
KE038 v1	0.78	Pos	0.1	Neg	1.93	Pos	1.55	Pos
KE038 v6	0.75	Pos	0.12	Neg	1.98	Pos	1.56	Pos
KE038 v12	0.88	Pos	0.14	Neg	2	Pos	1.48	Pos
KE038 v15	0.91	Pos	0.14	Neg	2	Pos	1.56	Pos
Patient no 40								
JH040 v1	0.4	Pos	0.12	Neg	1.31	Pos	1.72	Pos
JH040 v6	0.35	Pos	0.11	Neg	1.31	Pos	1.63	Pos
JH040 v12	0.34	Pos	0.11	Neg	1.24	Pos	1.59	Pos
JH040 v15	0.37	Pos	0.14	Neg	1.2	Pos	1.44	Pos
Patient no 44								
AH044 v1	0.08	Neg	0.03	Neg	1.56	Pos	1.74	Pos
AH044 v6	0.08	Neg	0.03	Neg	1.6	Pos	1.76	Pos
AH044 v12	0.07	Neg	0.03	Neg	1.62	Pos	1.8	Pos
AH044 v15	0.07	Neg	0.03	Neg	1.54	Pos	1.74	Pos
Patient no 45								
SW47 scr	0.39	Pos	0.07	neg	1.37	pos	1.87	pos
SW47 v6	0.34	Pos	0.07	neg	1.23	pos	1.92	pos
SW47 v8	0.74	Pos	0.17	neg	1.76	pos	1.81	pos
SW47 v10	0.63	Pos	0.08	neg	1.27	pos	1.96	pos
SW47 v12	0.38	Pos	0.08	neg	1.34	pos	1.99	pos
SW47 v13	0.4	Pos	0.08	neg	1.44	pos	2.1	pos
SW47 v15	0.4	Pos	0.08	neg	1.37	pos	1.99	pos
SW47 v8 rep	0.74	Pos	0.16	neg	1.78	pos	1.88	pos
Patient no 48								
LD050 v1	0.45	Pos	0.8	Pos	1.62	Pos	1.97	Pos

Sample	OD405/650 EBV EA IgG	interpn	OD 405/650 VCA IgM	interpn	OD405/650 VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
LD050 v6	0.44	Pos	0.87	Pos	1.66	Pos	1.95	Pos
LD050 v12	0.44	Pos	0.79	Pos	1.65	Pos	1.96	Pos
LD050 v15	0.44	Pos	0.91	Pos	1.71	Pos	2	Pos
Patient no 49								
AJ048 v1	0.09	Neg	0.12	Neg	1.78	Pos	1.69	Pos
AJ048 v6	0.08	Neg	0.12	Neg	1.84	Pos	1.61	Pos
AJ048 v12	0.09	Neg	0.12	Neg	1.93	Pos	1.67	Pos
AJ048 v15	0.09	Neg	0.12	Neg	1.94	Pos	1.68	Pos
Patient no 50								
JP049 v1	0.09	Neg	0.07	Neg	2	Pos	1.76	Pos
JP049 v6	0.15	Neg	0.08	Neg	1.98	Pos	1.76	Pos
JP049 v12	0.17	Neg	0.07	Neg	1.93	Pos	1.74	Pos
JP049 v15	0.13	Neg	0.07	Neg	1.86	Pos	1.73	Pos
Patient no 52								
PY052 v1	0.13	Neg	0.05	Neg	1.5	Pos	1.6	Pos
PY052 v6	0.11	Neg	0.05	Neg	1.5	Pos	1.6	Pos
PY052 v12	0.09	Neg	0.04	Neg	1.5	Pos	1.6	Pos
PY052 v15	0.11	Neg	0.05	Neg	1.6	Pos	1.6	Pos
Patient no 54								
JM054 scr	1.15	Pos	0.03	neg	1.89	pos	1.92	pos
JM054 v2	1.14	Pos	0.03	neg	1.84	pos	1.89	pos
JM054 v5	1.1	Pos	0.04	neg	1.8	pos	1.85	pos
JM054 v6	1	Pos	0.03	neg	1.78	pos	1.86	pos
JM054 v8	1	Pos	0.04	neg	1.81	pos	1.88	pos
JM054 v10	1	Pos	0.04	neg	1.8	pos	1.92	pos
JM054 v12	1	Pos	0.04	neg	1.81	pos	1.87	pos
JM054 v13	1.1	Pos	0.05	neg	1.86	pos	2.04	pos
JM054 v15	1.1	Pos	0.05	neg	1.89	pos	1.97	pos
Patient no 56								
JW056 v1	0.28	Pos	0.1	Neg	1.4	Pos	0.83	Pos
JW056 v6	0.29	Pos	0.11	Neg	1.5	Pos	0.93	Pos
JW056 v12	0.28	Pos	0.13	Neg	1.5	Pos	0.83	Pos
JW056 v15	0.31	Pos	0.12	Neg	1.4	Pos	0.99	Pos
Patient no 59								
VM 58 v1	0.12	neg	0.04	neg	2	pos	1.94	pos

Sample	OD405/650 EBV EA IgG	interpn	OD 405/650 VCA IgM	interpn	OD405/650 VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
VM 58 v5	0.11	neg	0.04	neg	2	pos	1.96	pos
VM 58 v6	0.1	neg	0.04	neg	1.94	pos	1.9	pos
VM 58 v8	0.087	neg	0.04	neg	1.83	pos	1.83	pos
VM 58 v10	0.084	neg	0.04	neg	1.83	pos	1.82	pos
VM 58 v12	0.097	neg	0.037	neg	1.8	pos	1.75	pos
VM 58 v13	0.088	neg	0.034	neg	1.82	pos	1.79	pos
VM 58 v15	0.078	neg	0.032	neg	1.81	pos	1.78	pos
Patient no 60								
BY061 v1	0.4	Pos	0.03	Neg	1.8	Pos	1.9	Pos
BY061 v6	0.41	Pos	0.03	Neg	1.8	Pos	1.9	Pos
BY061 v12	0.37	Pos	0.02	Neg	1.8	Pos	1.9	Pos
BY061 v15	0.37	Pos	0.04	Neg	1.8	Pos	1.9	Pos
Patient no 61								
PH062 v1	0.08	Neg	0.02	Neg	0.83	Pos	1.6	Pos
PH062 v6	0.08	Neg	0.02	Neg	0.87	Pos	1.7	Pos
PH062 v12	0.08	Neg	0.02	Neg	0.86	Pos	1.7	Pos
PH062 v15	0.07	Neg	0.02	Neg	0.89	Pos	1.7	Pos
Patient no 62								
JB063 v1	0.21	Equiv	0.11	Neg	1.4	Pos	1.8	Pos
JB063 v6	0.2	Equiv	0.11	Neg	1.4	Pos	1.8	Pos
JB063 v12	0.21	Equiv	0.09	Neg	1.4	Pos	1.9	Pos
JB063 v15	0.21	Equiv	0.09	Neg	1.4	Pos	1.9	Pos
Patient no 69								
KS069 v1	0.13	neg	0.09	neg	1.3	pos	1	pos
KS069 v5	0.13	neg	0.08	neg	1.27	pos	1	pos
KS069 v6	0.12	neg	0.08	neg	1.27	pos	1	pos
KS069 v8	0.13	neg	0.07	neg	1.28	pos	1.2	pos
KS069 v10	0.19	equiv	0.08	neg	1.26	pos	1.2	pos
KS069 v13	0.23	equiv	0.077	neg	1.41	pos	1.3	pos
KS069 v15	0.18	equiv	0.078	neg	1.3	pos	1.2	pos
Patient no 71								
WH072 v1	0.13	Neg	0.04	Neg	0.81	Pos	1.5	Pos
WH072 v6	0.11	Neg	0.04	Neg	0.83	Pos	1.5	Pos
WH072 v12	0.11	Neg	0.04	Neg	0.93	Pos	1.5	Pos
WH072 v15	0.11	Neg	0.04	Neg	0.88	Pos	1.6	Pos

Sample	OD405/650 EBV EA IgG	interpn	OD 405/650 VCA IgM	interpn	OD405/650 VCA IgG	interpn	OD405/650 EBNA 1 IgG	interpn
Patient no 72								
JW071 v1	0.22	Pos	0.05	Neg	1	Pos	1.3	Pos
JW071 v6	0.22	Pos	0.06	Neg	1.1	Pos	1.3	Pos
JW071 v12	0.2	Pos	0.06	Neg	1.1	Pos	1.3	Pos
JW071 v15	0.24	Pos	0.05	Neg	1.2	Pos	1.3	Pos